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689. THE EFFECT OF VARIATIONS IN NUTRIENT INTAKE UPON THE YIELD AND COMPOSITION OF MILK

III. A NOTE ON EXPERIMENTAL METHODS

BY A. W. A. BURT

National Institute for Research in Dairying, University of Reading

The measurement of the responses of the dairy cow to differences in feeding depends on the reduction of experimental error to a level which allows adequate statistical estimation of the effects of treatment. Considerable between-cow variations in milk yield and composition, and in their rates of change with time, occur, and lack of available cattle and facilities usually prevents the use of large-scale replication as a means of reducing the corresponding experimental error. This has led to the development of methods of comparing treatments on a within-cow basis, using various types of changeover design (1, 2, 3, 4, 5, 6, 7, 8, 9) instead of randomized block designs.

The planning of any experiment involves the selection of the most appropriate experimental design, and a system of observation which strikes a reasonable balance between experimental accuracy and economy of labour. The present paper is based upon a series of changeover experiments on variations in winter diets, and two randomized block experiments on the supplementation of pasture diets of the dairy cow, which have been carried out at this Institute. In it, the effects of some variations in methods of recording and statistically analysing the observations of milk yield, milk composition and live weight usually associated with such experiments will be discussed, as will some of the points involved in the selection of appropriate experimental designs for particular purposes.

MATERIAL AND METHODS

The results of seven winter feeding experiments using the 3×3 latin-square design of Cochran *et al.* (2) were available. Detailed accounts of five of these have been published (Exp. 3 (10), Exps. 4–7 (11)). A brief description of Exps. 1 and 2 has been given by Rowland (12). Eighteen cows were used in each experiment to compare three treatments, that is three complete replications of the design of Cochran *et al.* (2). Each experimental period lasted 5 weeks' in Exps. 1 and 2, and 3 weeks' in the remaining experiments. Milk yields were recorded at each milking, and milk samples for the determination of fat and solids-not-fat percentages were taken at four consecutive milkings, and bulked in proportion to the respective yields for subsequent analysis. These samples were taken in each week of Exps. 2–5, in the last 4 weeks of each period in Exp. 1, and in the last week of each period in Exps. 6 and 7. Live weights were recorded weekly and on three consecutive days at the end of each period in Exps. 1 and 2, and weekly and on the last 2 days of each period in Exps. 4 and 5. No weighing facilities were available in the remaining experiments.

In addition, some use was made of the results of two trials, using randomized block

designs, which were set up to study the responses of the grazing dairy cow to supplementary concentrates. In Exp. A, thirty-three cows were used to compare three treatments in an experimental period lasting 5 weeks, which followed and preceded control periods of 2 weeks' duration. In Exp. B, eighteen cows were used to compare the effects of two treatments in a trial lasting 2 weeks, which was followed and preceded by control periods of 1 week.

RESULTS

The effect of repeated observations upon experimental error

Milk yield. The effects upon experimental error, of using milk yields from the last week as compared with the last 2 weeks of each experimental period are shown in terms of the error coefficient of variation, and the minimum significant difference ($P=0{\cdot}05$), in Table 1. Mean minimum significant differences (lb./cow/day) were 1·3 lb. for data from 1 week and 1·2 lb. for data from 2 weeks. In three of the seven experiments, the inclusion of data from an additional week had no beneficial effect upon experimental error. Most of the observed reduction in error occurred in those experiments (1, 2 and 6) which showed high coefficients of variation by both methods.

Table 1. *The effect of repeated observations upon errors in milk yield*

	Yields from 1 week			Yields from 2 weeks		
Exp.	Error coefficient of variation (%)	Minimum significant difference (lb./cow/day)	Mean yield (lb./cow/day)	Error coefficient of variation (%)	Minimum significant difference (lb./cow/day)	Mean yield (lb./cow/day)
1	11·0	1·5	19·9	8·9	1·2	20·1
2	9·0	1·1	18·5	7·5	1·0	19·0
3	4·9	1·5	44·4	4·5	1·4	44·9
4	4·3	1·2	41·3	4·2	1·2	41·8
5	5·8	1·0	24·1	6·0	1·0	24·5
6	9·8	1·6	22·7	8·9	1·4	22·8
7	4·4	0·9	28·3	5·0	1·0	28·1
Mean	7·0	1·3	25·6	6·4	1·2	25·9

Table 2. *The effect of repeated observations upon errors in fat percentage*

No. of observations ...	1			2			3		
Exp.	Error coefficient of variation (%)	Minimum significant difference (%)	Mean fat (%)	Error coefficient of variation (%)	Minimum significant difference (%)	Mean fat (%)	Error coefficient of variation (%)	Minimum significant difference (%)	Mean fat (%)
1	4·1	0·11	3·82	3·6	0·09	3·79	3·0	0·08	3·74
2	6·7	0·17	3·67	4·8	0·12	3·62	4·3	0·11	3·62
3	22·9	0·56	3·60	8·7	0·42	3·60	—	—	—
4	7·9	0·20	3·67	4·6	0·12	3·63	—	—	—
5	4·7	0·13	3·87	3·9	0·11	3·83	—	—	—
Mean	9·3	0·23	3·73	5·1	0·17	3·69	—	—	—

Fat percentage. The effects of using fat percentages measured in 1, 2, or 3 successive weeks at the end of each experimental period are shown in Table 2. Mean minimum significant differences decreased from 0·23 to 0·17 unit of percentage when data from 2 weeks were used. The inclusion of data from a third week in Exps. 1 and 2, which had experimental periods of 5 weeks duration, produced a further reduction in minimum significant differences of 0·01 unit of percentage in each case.

Table 3. *The effect of repeated observations upon error in solids-not-fat percentage*

No. of observations ...	1			2			3		
Exp.	Error coefficient of variation (%)	Minimum significant difference (%)	Mean solids-not-fat (%)	Error coefficient of variation (%)	Minimum significant difference (%)	Mean solids-not-fat (%)	Error coefficient of variation (%)	Minimum significant difference (%)	Mean solids-not-fat (%)
1	1·5	0·09	8·54	1·3	0·08	8·54	1·3	0·07	8·53
2	1·6	0·09	8·39	1·1	0·06	8·40	0·8	0·05	8·41
3	1·4	0·08	8·40	1·2	0·07	8·42	—	—	—
4	1·6	0·09	3·31	1·4	0·08	8·31	—	—	—
5	1·6	0·09	8·41	1·3	0·07	8·42	—	—	—
Mean	1·5	0·09	8·41	1·3	0·07	8·42	—	—	—

Table 4. *The effect of using weights recorded upon* 1, 2 *or* 3 *successive days upon errors in live weight*

Weights on ...	1 day		2 days			3 days	
Exp.	Error coefficient of variation (%)	Minimum significant difference (lb.)	Error coefficient of variation (%)	Minimum significant difference (lb.)	Mean live weight (lb.)	Error coefficient of variation (%)	Minimum significant difference (lb.)
1	1·7	13·6	1·8	14·1	1177	1·7	13·6
2	1·4	10·5	1·4	11·0	1142	1·5	11·4
4	1·6	12·0	1·6	12·5	1132	—	—
5	1·9	13·9	1·3	9·5	1081	—	—
Mean	1·7	12·5	1·5	11·8	1133	—	—

Solids-not-fat percentages (Table 3). The mean effect of using observations recorded in 2 weeks instead of 1 was to reduce the minimum significant difference from 0·09 to 0·07 percentage unit. In fact, the reduction was 0·01 percentage unit in four of the five experiments. The effect of the addition of data from a third week in Exps. 1 and 2 was to reduce minimum significant differences by a further 0·01 percentage unit.

Live weight. The effects of using live weights recorded on the first, first and second, and first, second and third days of weighing at the end of each period, upon experimental error, are shown in Table 4. In three out of four experiments the minimum significant differences tended to increase slightly with increasing number of weighings. In Exp. 5, however, the use of a second day's live weights diminished error appreciably.

Development of treatment responses with time

In Exps. 1, 2, 4, 5 and 7, mean milk yields on one treatment were significantly different from those on the remainder. This difference was computed separately for the first and subsequent weeks of all experimental periods in each experiment with the results shown in Table 5. In all cases there was a marked increase in treatment differences between the first and second weeks. Thereafter, there was no further increase in the third week of Exps. 4, 5 and 7, but the difference increased slightly and consistently throughout the remainder of the 5-week experimental period in Exps. 1 and 2. This difference between the two sets of experiments may be connected with the levels of feeding used, since the normal feeding standards (13) were the basis of the best treatment in Exps. 1 and 2 and the worst in the remainder. In addition, individual rations were changed in accordance with changes in yield in Exps. 1 and 2, but remained constant throughout each experimental period in Exps. 2, 4, 5 and 7.

Table 5. *Changes in the response of milk yield to treatment with time*

	Weeks of experimental period				
	1	2	3	4	5
Exp.	Mean difference (lb./cow/week)				
1	+8·2	+16·8	+17·1	+20·2	+24·5
2	+3·4	+19·9	+22·5	+24·6	+29·6
4	+12·0	+18·4	+14·5	—	—
5	+4·1	+10·5	+10·6	—	—
7	+6·0	+12·9	+11·0	—	—

Table 6. *Change in the response of solids-not-fat percentage to treatment with time*

	Weeks of experimental period				
	1	2	3	4	5
Exp.	Mean difference (%)				
1	—	+0·10	+0·19	+0·21	+0·22
2	+0·01	+0·03	+0·06	+0·06	+0·10

Similar data in conjunction with significant treatment effects were available for solids-not-fat percentage in Exps. 1 and 2. Differences between treatment means increased markedly up to the third week in both experiments (Table 6), but less rapidly thereafter.

The analysis of randomized block experiments

Three different methods of analysis of the effects of treatment upon milk yield were compared using the data from Exps. A and B (Table 7). The methods were (*a*) analysis of variance of milk yield in the latter part of the experimental period, (*b*) the analysis of covariance of milk yield in the latter part of the experimental period with milk yield in the previous control period, and (*c*) the analysis of variance of individual responses in yield measured as the difference between milk yield in the latter part of the experimental period and the mean yield in the two control periods. In Exp. A, milk yields in the last 2 weeks of the experimental and control periods were used, and milk yields from the last week of each period in Exp. B.

Table 7 shows that covariance analysis with initial yield reduced error standard deviations and minimum significant differences slightly in both experiments, but that the analysis of individual responses rather than individual yields was much more effective in

Table 7. *Comparison of three methods of analysis of milk yields in randomized block experiments*

Method ...	Analysis of variance of Y	Analysis of covariance of Y with X_1	Analysis of variance of responses $(Y-\frac{1}{2}[X_1+X_2])$
Exp. A:			
Error standard deviation (lb./day)	4·5	4·0	1·8
Minimum significant difference (lb./day)	4·0	3·6	1·6
Exp. B			
Error standard deviation (lb./day)	2·2	2·0	1·6
Minimum significant difference (lb./day)	2·2	2·0	1·6

N.B. X_1 = Milk yield in the pre-experimental control period.
X_2 = Milk yield in the post-experimental control period.
Y = Milk yield in the experimental period.

reducing error. Covariance analysis reduced minimum significant differences by 10% of the value for analysis of variance of experimental milk yields in each case, while the percentage reductions due to the analysis of variance of the responses were 60 and 27% for Exps. A and B respectively.

DISCUSSION

Tables 1–3 show that the mean minimum significant differences in 3×3 latin-square designs using eighteen cows were 1·3 lb. milk, and 0·23 and 0·09 percentage unit of fat and solids-not-fat respectively, when data from the last week of each experimental period were used in the analysis. The use of data from 2 weeks reduced these significant differences by 0·1 lb. milk, and 0·06 and 0·02 percentage unit of fat and solids-not-fat, respectively, while the addition of observations from a third week reduced the minimum significant difference by 0·01 percentage unit of fat and solids-not-fat in Exps. 1 and 2. It must be stressed that these estimates apply only to one particular experimental design and system of recording observations, since the improvement in accuracy must obviously depend upon the relative importance of within-cow random error in the total experimental error. However, this improvement may be expected to be relatively more important in change-over trials, where the final experimental error is on a within-cow basis, than in randomized block experiments where this error usually includes a large component due to differences between cows.

The limited data on the responses of milk yield to treatment with time agree with the conclusions of other workers (14,15) in showing that the maximum response to a ration is practically fully developed within 2 weeks of the start of feeding, but that the full response to underfeeding may take rather longer to develop as the cows reserves become depleted. In fact, this may affect the application of the results of short-term trials to farm practice, since such trials may estimate the full effects of an increase in the ration above normal standards upon milk yield (although not on live-weight), but underestimate the full effects of poor treatment.

The full effects of treatment upon solids-not-fat percentage took rather longer to develop than those on milk yield. This may have some influence on the application of the effect of repeated observations upon experimental error, since increasing the number of weeks used in the analysis may reduce the observed effects of treatment as well as their associated error. In fact, the mean results of Exps. 1 and 2, with respect to solids-not-fat percentage, showed that increasing the weeks of observation from one to three per period, reduced both the mean differences between treatments and the mean minimum significant difference by 0·02 percentage unit, and therefore resulted in no increase in relative efficiency. The limited data on factors affecting errors in studies of milk composition, presented in this paper, stress the need for further studies using other experimental designs and distributions of samples.

Recording live weights on more than 1 day failed to reduce error in three out of four experiments. Although it is generally agreed that 3-day weights should have appreciably lower experimental errors than single weights (16,17), increasing errors with successive weighings have been observed in certain circumstances (18,19,20). Much of the variation in individual live weights may be associated with contemporary variation in the average of the experimental group, as observed in some instances by Lush, Christensen, Wilson & Black (17) in which case the improvement due to repeated weighing would be expected to

be very small. This point applies equally to other experimental observations. Repeated weighing would be expected to be more effective in reducing error, than in the experiments described above, when the cows in a block are weighed on different days, and less effective in randomized block experiments where much of the error is usually associated with differences between animals (21).

In randomized block experiments, errors in the analysis of variance of the milk yields recorded at the end of the experimental period are due to differences between cows in initial yield which are not accounted for by the arrangement of cows in blocks, to differences arising from between-cow variation in the rate of change in yield in the experimental period which are inherent and not due to treatment, and to random error. Covariance analysis removes the effects of differences in initial yield, but the analysis of individual responses measured as the difference between yield in the experimental period, and mean yield in pre- and post-experimental control periods takes account of differences in initial yield and inherent differences in its rate of change. The effect of this type of analysis in reducing experimental error is shown in Table 7. It is of interest to note that the minimum significant difference in the analysis of variance of individual responses was the same (1·6 lb./cow/day) in Exps. A and B which had practically similar degrees of replication (11 and 9 per treatment, respectively), although the errors in the analyses of variance and covariance of milk yields in the experimental period were much greater at the end of the 5-week experimental period of Exp. A than the 2-week experimental period of Exp. B. This illustrates the expected increase in error due to individual differences in rate of change of yield with increasing length of experimental period.

The effects of analysis of individual responses, compared with the more usual analysis of variance or covariance of experimental observations, depend upon the relative importance of individual secular trends as sources of error. In milk composition data from short term experiments, such individual trends may be expected to be unimportant relative to random error. In fact, the application of the method to the very limited solids-not-fat data available from one experiment, indicated that it was far less effective in reducing errors than in the corresponding analysis of milk yield.

This type of analysis is essentially a variation of the switchback design in which each cow receives two treatments, one applied in the first and last experimental period, and the other in the intervening period, which has been described in detail by Lucas (8) and found to be a particularly effective design. It is also the method used in the classic Danish group feeding trials (22), without the corresponding statistical analysis of error.

The residual effects of treatment in the period following its application may be estimated in the latin-square design of Cochran *et al.* (2), but not in the switchback design described by Lucas (8). However, these seem likely to be unimportant, if the data from the first 1 or 2 weeks of each experimental period is omitted from the analysis, since in none of the analyses summarized in Tables 1–4 inclusive were there any significant residual effects of treatment. In randomized block trials when the results are analysed as individual responses, the effect of treatment residuals in the final control period will be usually to reduce the measured responses to treatment. Any marked residual effects may, however, be readily estimated free from the complications of the application of further treatment differences, and can probably be eliminated by extending the final control period.

The computed effects of varying the degree of replication upon minimum significant differences in milk yield in 3×3 latin-square designs and randomized block experiments are shown in Table 8, based on the mean value of seven trials with eighteen cows in Table 1, and the analysis of variance of individual responses in Exp. A (Table 7). The computed values for 3×3 trials allow for the expected change in error standard deviation in the latin-square design, due to the relative constancy of within-cow errors and the disproportionate increase in error degrees of freedom with increasing replication (6), and for the usual effects of replication upon the standard errors of treatment means and upon the

Table 8. *The effect of replication upon minimum significant differences in milk yield in experiments with three treatments*

(*a*) 3 × 3 latin square		(*b*) Randomized block (analysis of variance of individual responses)	
No. of cows	Estimated minimum significant difference (lb./cow/day)	No. of cows	Estimated minimum significant difference (lb./cow/day)
6	2·9	18	2·3
12	1·6	24	1·9
18	1·2	30	1·7
24	1·0	33	1·6
		36	1·5
		42	1·4
		48	1·3

values of '*t*' at $P = 0{\cdot}05$. Table 8 shows that little is gained by increasing the number of cows beyond eighteen in 3×3 latin squares, and that approximately three times the number of cows are needed in randomized block experiments to achieve similar minimum significant differences. However, it must be remembered that these estimates are only approximate, and are probably more reliable for 3×3 latin squares than for randomized blocks. The latter are based on one summer grazing trial with no control of individual roughage intakes, and the difference in favour of the latin-square design may therefore be slightly overestimated.

Although randomized block experiments have much higher errors than changeover trials with a similar number of cows, the former design is essential in certain types of experiment, e.g. where the total treatment period must be too short to allow the completion of a changeover design, or where its extension would involve a serious risk of treatment x period interactions which are assumed to be absent in the analysis of changeover trials, or where the treatments being studied must be applied during a specified period of the cow's lactation cycle. In addition, the randomized block has some advantages, as it allows the straightforward estimation of the residual effects of treatment and multiple regression analysis of factors affecting individual responses to treatment. The latin square and similar designs (6) give low errors and allow some estimation of residual effects, although their errors may be biased in certain circumstances (5,6,7), while the switchback design of Lucas (8) gives even lower errors but assumes that residual effects are absent.

SUMMARY

1. Data from seven winter feeding experiments with dairy cows, using 3 × 3 latin-square designs and from two randomized block experiments on the supplementation of summer grazing, were used in a study of some aspects of experimental technique.

2. In the 3 × 3 trials the use of observations from 2 weeks instead of the last week of each period reduced minimum significant differences by 0·1 lb. milk/cow/day, and 0·06 and 0·02 mean percentage unit of fat and solids-not-fat, respectively.

3. In three out of four experiments no advantage was obtained from recording live weights on more than 1 day at the end of each period.

4. It was shown that the analysis of variance of milk yields expressed as individual differences between yield in the experimental period, and the mean of pre- and post-experimental control periods was considerably more efficient than that of yields in the experimental period, or covariance with yield in the initial control period.

5. The results were discussed in relation to the sources of error involved, and the relative efficiency of various types of experimental design.

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690. AN INVESTIGATION OF FOURTEEN HERDS PRODUCING SUBSTANDARD MILKS IN THE EAST OF SCOTLAND

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Rowland (1) in 1947 gave average fat and solids-not-fat (S.N.F.) figures for milks produced in the Reading area in the years 1941–45, of 3·55 % fat and 8·65–8·70 % S.N.F. Provan & Jenkins (2) in 1948 gave average figures of about 3·55 and 8·62 % S.N.F. for milks received at some Milk Marketing Board Creameries in north-western areas of England during 1946. Both Provan (2, 3) and Rowland (1, 4) have pointed out that a decline in the compositional quality of milk has taken place in the past 30 years in England and Wales. More recently Provan (5) gave average figures of 3·60 % fat and 8·70 % S.N.F. for milks being produced in England and Wales during 1955, but pointed out that since these figures included values for milks produced in Devon and Cornwall they gave an optimistic picture of the quality of the milk being produced in the area as a whole. He also confirmed the strong seasonal effect on milk composition, and the variation in this effect in different parts of the country which he noted in 1951.

Tocher (6) in 1925 gave 3·95 and 8·80 % as the average percentages for fat and S.N.F. in 676 samples of milk collected from representative dairying areas in Scotland in 1921–22. In the Report of the Working Party on Quality Milk Production (7), it is stated that:

'Evidence submitted from Scotland showed that for the past twenty years the composition of the milk supply had remained fairly steady at a high level. If anything there has been an improvement in the last year or so.'

Chalmers (8) gave values of 8·69 % S.N.F. for the south-west and the east-central regions of Scotland in the period August to January, and 8·74 % for the south-west and 8·73 % for the east-central areas of Scotland for the period February to July.

Despite the encouraging nature of these statements a considerable number of herds are met with in advisory work in the south-east of Scotland in which there is difficulty in maintaining a reasonable S.N.F. level, especially at certain times of the year. The serious nature of the problem is well illustrated by the example of a large city creamery in the south-east of Scotland, where in the late summer 50 % or more of the incoming milks may fall below the 8·50 % S.N.F. level. The problem of poor quality milk production in the south-east of Scotland is more serious than one would expect from published work.

EXPERIMENTAL

The present investigation was undertaken to examine differences in the detailed composition of normal and substandard milks, and to assess the extent to which such factors as feeding, mastitis and stage of lactation were responsible for poor milk quality. To this end fourteen herds with a past history of poor quality milk were examined. Milk samples from each animal were taken from successive morning and evening milkings, and bulked according to yield to give a single sample for each cow. The samples were analysed for fat,

total solids, crude protein, lactose and chloride. Ash contents were obtained by difference. While sampling the milk, data were collected whenever possible concerning the feeding, incidence of mastitis, stage of lactation, age of cow and date of successful service for each cow. The sampling was made during August and September, i.e. in a period when in this area the greatest lowering of the S.N.F. content has been observed to take place. The cows were at grass and most were receiving some supplementary feeding. For most herds no accurate information on feeding was available, but where it was obtained the rationing appeared to be adequate judging by the usually accepted Woodman standards. Information concerning the incidence of mastitis was also difficult to obtain, and in fact only a very few cases of a severe clinical type were reported. No information concerning past infections or present cases showing occasional clots or like symptoms were reported.

METHODS OF ANALYSIS

(1) Fat content was determined by the Gerber Method (B.S.S. no. 696, 1936).

(2) Total solids content was determined by a gravimetric procedure involving evaporation of 5 g. milk on a water-bath with further drying in an oven for 3½ hr. at 98–100° C.

(3) Lactose was determined iodometrically by the method of Hinton & Macara (9) with a modified clarification technique using potassium ferrocyanide and zinc acetate. No correction was made for the volume of the precipitate in the final calculation, since according to Gorbach & Schäfer (10) it is unnecessary.

(4) Crude protein was determined by estimating total nitrogen content by a semi-micro-Kjeldahl procedure and multiplying the result by 6·38.

(5) Chloride was determined by the wet oxidation method of Davies (11).

RESULTS AND DISCUSSION

Four hundred and sixteen animals were involved in the investigation. An evening and a morning sample were taken for each cow and mixed in proportion to the yields at the two milkings, giving 416 samples in all. Those samples that had a S.N.F. content of 8·50% or over were classified as normal, and those with a S.N.F. content below 8·50% as poor or substandard milks. The choice of this S.N.F. figure was purely arbitrary, and was based on the fact that in the past few years the term 'poor milks' has come to mean those that fail to meet the legal presumptive standard for S.N.F. The 416 samples in the present investigation were made up of 278 normal samples and 138 substandard samples.

Table 1. *Average values for the composition of the milk* (%)

	All 416 samples	Normal samples	Substandard samples
Fat	3·96	4·16	3·57
S.N.F.	8·76	9·07	8·14
Crude protein	3·45	3·63	3·09
Lactose	4·54	4·65	4·32
Ash	0·77	0·79	0·73
Chloride	0·113	0·106	0·127

The average values for the various milk constituents for all the data are given in Table 1 together with average figures for the normal and substandard groups.

The difference in the S.N.F. values between the normal and the substandard groups was 0·93%, 35·5% of this difference being due to a drop of 0·33% in the lactose content and

58·1% to a drop of 0·54% in crude protein. The remaining 6·4% of the difference must be allocated to changes in the ash constituents and those constituents of the S.N.F. other than lactose and protein. It is well to bear in mind that this value of 6·4 includes the errors involved in determining the S.N.F., lactose and protein. The substandard group showed a much lower fat content than the normal group and a considerably higher chloride content.

There was considerable variation in the quality levels of the herds examined, and it was decided that a fairer comparison between the normal and substandard milks could be obtained if they were compared within the individual herds, and the differences in milk constituents so obtained used to calculate differences for the whole population. However, such within-herd comparisons were not of equal value in each herd. Thus, in one of the herds only three substandard milks were compared with thirty-six normal samples, and this was obviously not as good a comparison as another where sixteen in each group were available. The within-herd differences in milk constituents between the normal and substandard groups were thus weighted in arriving at figures for the whole of the data. The figures so obtained are presented in Table 2.

Table 2. *Differences in the percentage of milk constituents present in normal and substandard milks*

	Values for normal milks minus the corresponding values for substandard milks (%)
Fat	+0·53
S.N.F.	+0·88
Crude protein	+0·46
Lactose	+0·38
Ash	+0·04
Chloride	−0·025

The difference in S.N.F. between the two groups is here slightly less than that shown in Table 1. The relative importance of lactose and protein is also different, with lactose playing a greater part than it appeared to play in Table 1. Thus 43·2% of the reduced S.N.F. content is here due to a drop in lactose, while 53·2% of the fall in S.N.F. is due to a drop in crude protein. The remaining 3·6% of the S.N.F. reduction is allocated to the ash constituents, but for reasons already given this figure does not give a true indication of the importance of the ash constituents in helping to lower the S.N.F. content of the substandard samples.

According to Rowland (1), limitation of either the starch or the protein equivalent fed to cows can bring about a decreased S.N.F. content in the milk produced, due very largely to a decline in the crude protein content. Holmes, Waite, MacLusky & Watson (12) have shown that feeding starch equivalent at levels above the usually accepted standards can lead in some circumstances to the production of milk with an increased S.N.F. content, which is due largely to an increase in the crude protein content of the milk.

It has been shown by Rowland (13) that the S.N.F. content of milk may be considerably reduced by the presence of a mastitic condition of the udder even where the condition is subclinical. This drop in S.N.F. is due almost entirely to a fall in the lactose content of the milk.

Because of lack of sufficient accurate information concerning feeding and the incidence

of mastitis in the cows examined, it was decided to use the above facts in an attempt to assess the relative importance of feeding and mastitis in causing the lowered S.N.F. in the substandard group. Thus, it is suggested that if this drop had been due to a lowering of the protein content it would be reasonable to assume that the drop was caused largely by inadequate feeding. On the other hand, if the S.N.F. drop had been due to a lowered lactose then it might be reasonable to assume that mastitis was the main cause. Other factors would effect the lactose and crude protein levels, but probably none of them would have so marked an effect as stage of lactation. Here the low protein stage of lactation is associated with a high lactose content (14). Therefore, milks with a lowered S.N.F. content due to a stage of lactation effect and having a lowered protein would show normal, or even higher than normal, lactose contents. In the present work most of the average drop in S.N.F. was due to a fall in the protein, but this was accompanied also by a considerable fall in lactose. It is felt that this may be taken as a good indication that in the milks studied here mastitis played an important part in lowering the S.N.F. content. The high chloride figure for the substandard samples lends support to this contention. Only in early or late lactation milks is there normally an increased chloride content, and this is accompanied by increased and not decreased S.N.F. levels as were observed here.

Rowland (4) states that the decline in S.N.F. over the past 25 years is due almost entirely to a fall in the protein content of the milk. The very important part played by the lowering of the lactose levels in the present population is not in agreement with this. Provan (5) and Ling (15) are of the opinion that mastitis is not a serious factor in reducing the national level of milk S.N.F., but it appears probable that in the population examined in the present work mastitis was playing a very important part. Several herds in this area, that have been producing poor quality milk recently, have been shown to have a high proportion of subclinical mastitis, which may be more important in lowering the national S.N.F. level than is generally thought.

At the time of the present investigation the cows were at grass which was classified as being of very good quality. At the same time there were observations that stock was not thriving as well as might be expected. It is quite likely that farmers were being somewhat optimistic in their assessment of the value of the grass. Be this as it may, only four of the herds here dealt with were receiving supplementary feeding according to milk yield. Six others were receiving a little cake or a handful of dairy nuts at milking time, while the remainder were receiving no extra food whatever. The work of Holmes (12) has shown the need for feeding higher energy levels for milk production, and Sjollema (16) has shown the need for higher maintenance allowances for cows on pasture. In view of these facts it is not unlikely that a large proportion of the cows in the investigation were in fact not receiving all the food they required. This, in conjunction with the much lowered crude protein contents of the substandard samples, indicates that feeding was a major factor in causing the lowered S.N.F. in the substandard group of this population.

Many other factors, apart from the two major ones of feeding and mastitis, affect the composition of milk, and the effect of certain of these other factors was examined in the present investigation.

Effect of breed

The cows were all either of Ayrshire or Friesian descent. A few crosses of the two breeds and a few Ayrshire/Shorthorn crosses were met with, and these were included with the Ayrshire group in the following discussion. The average S.N.F. of this group was

8·87 % as compared with the figure of 8·58 % for the Friesian group. Of 218 cows in the Ayrshire group, 59 or 27·1 % yielded substandard milk, and of the 130 cows in the Friesian group 52 or 40 % gave substandard milk.

The Friesian type group formed 37 % of the population and obviously had a considerable effect on the average S.N.F. content. Thus, the overall S.N.F. average was 8·76 % a lowering of 0·11 % compared with the potential 8·87 % if the cows had all been of the Ayrshire type.

Effect of age

Tocher (6) showed that the regression of S.N.F. on age of cow was linear. Provan (5) considers that the S.N.F. content of milk from cows in their fourth lactation may be as much as 0·2–0·4 % below that of the milk in the first lactation. In the present investigation the age and lactation number of the cows were recorded. The proportion of the cows of different ages giving substandard milk is shown in Table 3.

Table 3. *Age of cow and the proportion of milks that were substandard*

Age of cow (years)	No. of milk samples analysed in each age group	Percentage of samples with S.N.F. below 8·5 %
Up to 4	112	23·2
4–5	106	25·4
5–6	53	37·7
6–7	62	37·1
7–8	40	50·0
8–9	11	45·4
9 and over	32	56·2

Three age groups became obvious, differing in the proportion of milks which were substandard. Thus, the first group comprising animals up to 5 years of age showed about 25 % of substandard samples. The cows that were between 5 and 7 years of age showed about 37 % of substandard samples, while those that were over 7 years of age showed up to 56 % of substandard samples.

Table 4. *Lactation number and the proportion of milks that were substandard*

Lactation no.	No. of milk samples analysed	Percentage of samples with S.N.F. below 8·5 %
1	104	20·2
2	125	25·6
3	60	38·3
4	67	40·3
5	30	60·0
6	20	55·0
7	6	66·7
8	4	50·0

Table 4 showed a similar situation where lactation number and proportion of substandard samples were examined. Three groups were again obvious. The first consisted of first and second lactation cows, the second of third and fourth lactation cows and the third of cows in lactation later than the fourth. A considerable increase in the proportion of substandard samples occurred in the second group compared with the first and this increase was even more more serious in the third group.

This evidence supports the view expressed by Provan (5) that the increase in herd life

of the dairy cow during the last three decades may be partly responsible for the reported decline in S.N.F. content of the national milk supply. Certainly it would appear that in a herd with a high proportion of cows in their fifth or later lactation there may be difficulty in maintaining a satisfactory S.N.F. level.

In the present investigation the average S.N.F. of the milk of the cows other than those in their fifth or later lactation was 8·83%, while the figure for the whole of the population was 8·76%, a lowering of 0·07% which would have been avoided if the older cows had been eliminated.

Effect of stage of lactation

Tocher (6) considered that the S.N.F. content of milk is at a minimum between the 100th and 190th day of the lactation. Waite (14) considers that the S.N.F. falls to a minimum at 45 days, with a slow rise to the 135th day, when the level remains constant until the last 2 months when it rises sharply. The percentage of samples in the present investigation that were below 8·5% S.N.F. for cows at various stages of lactation is given in Table 5.

Table 5. *Stage of lactation and the proportion of milks that were substandard*

Stage of lactation (days)	No. of milk samples analysed	Percentage of samples with S.N.F. below 8·5%
30	46	28·3
60	25	56·0
90	26	50·0
120	30	26·7
150	54	29·6
180	54	38·9
210	39	41·0
240	34	29·4
270	43	47·1
300	39	25·6
315	24	29·2

The greatest proportion of substandard samples occurred in the milks of those cows that were between the 45th and 105th day of lactation which is what one would expect from the work of Waite (14). However, two other periods of lactation, viz. between 165–225 days and between 255 and 285 days also showed relatively high proportions of substandard samples. The occurrence of these periods so late in lactation is difficult to reconcile with any published work. According to Waite (14) the S.N.F. content at this period should be rising sharply so that this period would not be regarded as critical for the occurrence of low S.N.F. values.

The effect of removing those cows that were in the critical stages of lactation from the present population was assessed. The overall average of 8·75% S.N.F. would have been raised to 8·86% by this means, assuming the cows were replaced by animals giving milk of similar S.N.F. content to that of the rest of the population.

It is considered that much could be done to maintain satisfactory S.N.F. levels by staggering services and therefore eliminating the bunching of calvings. This would ensure that the herd would never have a high proportion of cows at the same stage of lactation. In a recent example a herd of thirty cows was giving an average S.N.F. in late March of 8·30%. On investigation it was found that twenty of the cows had calved during January and early February. The herd showed no signs of mastitis and the feeding was adequate. It would appear that the low S.N.F. level in this herd resulted mainly from a stage of

lactation effect. Experience indicates that this factor is of greater importance than is generally realized as a cause of both low S.N.F. and low fat milks especially at certain times of the year.

Effect of length of interval between calving and successful service

The percentage of samples that gave milk with less than 8·5% S.N.F in relation to the length of interval between calving and successful service is shown in Table 6.

Table 6. *Length of interval between calving and successful service and the percentage of milks that were substandard*

Days between calving and successful service	No. of samples	Percentage of samples with S.N.F. below 8·5%
Up to 30	69	29·0
31–60	83	32·5
61–90	98	42·9
91–120	36	38·9
121–150	28	35·7
151–180	17	35·3
181–210	16	56·3
211–240	12	50·0
241–270	22	22·7

A higher proportion of substandard milk was found with those cows that had an interval between calving and successful service of between 195 and 255 days. Those with an interval greater than 255 days showed fewer substandard samples than any other. The evidence obtained is too indefinite for any conclusion to be reached on the effect of this factor.

CONCLUSIONS

The lowering of the S.N.F. in the milk of the present cow population was due almost equally to a lowering of the lactose and crude protein contents. It is considered that the lowered lactose was due to mastitis and the lowered protein to inadequate feeding, and that these two factors played almost equally important parts in the lowering of the S.N.F. content of the substandard milks.

For animals older than 7 years or in a lactation later than the fourth, there was a greatly increased proportion of substandard milks. Stage of lactation also affected the proportion of substandard samples produced, the proportion being especially large during the period between the 45th and 105th day.

The Friesian type cows in the population showed a greater proportion of substandard samples than the Ayrshire type group, although the proportion in the latter, i.e. 27·1%, was by no means small.

Attention paid to adequate supplementary feeding in the late summer and to the early detection and control of mastitis could do much to maintain the S.N.F. content of milk at a satisfactory level. At the same time the position could be improved by limiting the number of older cows in the herd and by staggering calvings. It is especially important to ensure that a large proportion of the herd is not at a critical stage of lactation at that season of the year when trouble in maintaining S.N.F. levels is greatest.

SUMMARY

1. Four hundred and sixteen individual cow samples of milk from fourteen herds were analysed for fat, total solids, crude protein, lactose and chloride.

2. Wherever possible information concerning feeding, incidence of mastitis, age of cow, stage of lactation and successful service dates was collected.

3. The milk samples were divided into normal (with 8·50% S.N.F. or over) and substandard (with less than 8·50% S.N.F.) groups.

4. The difference in S.N.F. content between the normal and substandard groups was due almost equally to a drop in lactose and protein, but with the latter playing the major part. Thus, although inadequate feeding was probably a major factor in lowering the S.N.F. in this population the fact that the lactose content was so often low was taken as indicating that mastitis was also important.

5. Breed, age of cow and stage of lactation all affected the incidence of low S.N.F. milks.

6. On average the substandard milks showed a reduced fat content as well as a lowered S.N.F. level.

The author thanks his colleagues of the County Advisory Staffs who sampled the milks and collected the very detailed information concerning individual animals, and also Miss M. E. Simpson for valuable technical assistance.

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691. OXYGEN CONSUMPTION AND LACTOSE SYNTHESIS RATES OF MAMMARY GLAND SLICES FROM LACTATING GUINEA-PIGS

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(With 1 Figure)

INTRODUCTION

The relationship between changes in mammary structure and lactational performance has been discussed and investigated by the present author and his colleagues (Naito (1); Naito, Shoda & Nagai (2)). Other workers have demonstrated by the *in vitro* tissue-slice technique that various metabolic activities of mammary tissue *in vitro* are correlated with the physiological state of the gland (Folley & French (3,4,5) Kirkham & Turner (6); Hoover & Turner (7); Smith (8)). The respiratory activity of tissue from lactating animals observed by these workers did not directly indicate milk synthesis *in vitro*, although the R.Q. measurements did suggest that net fat synthesis was occurring. More direct evidence of synthesis of an essential milk constituent *in vitro* has come from the work of Grant (9); Knodt & Petersen (10); Malpress & Morrison (11); Hills & Stadie (12); Reithel, Horowitz, Davidson & Kittenger (13), and more recently by Heyworth & Bacon (14) and Duncombe (15), who have shown that lactose synthesis may be demonstrated in mammary tissue slices. The present communication describes a preliminary approach to the problem of studying quantitatively lactose synthesis *in vitro* in relationship to milk yield *in vivo*.

The work of Heyworth & Bacon (14) suggests that the guinea-pig is a better animal for studying lactose synthesis *in vitro* than the rat. Guinea-pigs were therefore chosen as experimental animals in the present study. Little is known of the shape of the lactation curve in this species, indeed there appears to be only the one report of Nelson, Kay, Moore, Williams & Herrington (16), so the first part of this communication is concerned with the construction of a lactation curve, and the second part with an investigation of the lactose synthesis and oxygen consumption of slices of mammary gland taken from guinea-pigs, in various stages of lactation.

EXPERIMENTAL

Animals. Female guinea-pigs of 500–900 g. weight were used and were fed *ad lib.* on the Medical Research Council's pellet diet No. 18. Within 10 hr. of parturition the number of young was reduced to two.

Estimation of milk yield. The young were separated from the mother by placing them in a small cage within the mother's cage. This was done because Nelson *et al.* (16) observed that complete separation out of the mother's sight was undesirable. Young were allowed

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to suckle for 30 min. four times daily in the first 2 days, three times daily in the following 6 days, and twice daily thereafter. Milk yield was determined by weighing the young before and after the suckling period.

Preparation of slices. The animals were killed by bleeding from the carotid artery after being stunned. The mammary glands were dissected off, weighed and placed in ice-cold Ringer III (Krebs (17)). The lateral portions of the gland were preserved for histological examination. From part of the central portion slices were cut approximately 0·5 mm. thick with the microtome described by Stadie & Riggs (18). The slices were washed in cold oxygenated Ringer III.

Incubation. Slices were prepared and 120 mg. portions were incubated in Ringer III containing 17 mM. glucose as substrate equilibrated with 100% O_2 as described by Heyworth & Bacon (14). Controls were similarly treated without incubation. Both incubated samples and controls were set up in duplicate.

Estimation of dry matter (DM). Aliquots of slices were dried at 110° C. for 24 hr. In preliminary experiments it was found that the dry-matter content of washed slices was similar to that of the finely minced and washed tissue used for DNA determination. Accordingly, slice dry-matter content only was determined for each animal.

Separation and estimation of lactose. After incubation, the slices were ground with fine sand in a solution of 1·0 ml. 1 mM. $HgCl_2$ to stop further enzyme activity and then centrifuged. The lactose in the supernatant was isolated chromatographically using 5·0 μl. aliquots on Whatman No. 1 chromatography paper (9 × 22·5 in.). The strips were developed in the descending developer in the solvent (ethyl acetate 3, glacial acetic acid 1, water 3) described by Jermyn & Isherwood (19), for 24 hr. at 25° C. after about 3 hr. equilibration in the developing-medium vapour. After development, the location of lactose was determined from guide spots appearing after spraying with benzidine-trichloracetic acid reagent (Bacon & Edelman (20)). In such a procedure the Rg was 0·1936. The portions of paper containing lactose were cut and eluted for 16 hr. in 3 ml. distilled H_2O at room temperature. After shaking, pressing the paper, and centrifuging down any paper fibres, the lactose in the supernatant was estimated by the method of Bath (21). To 1·0 ml. of the supernatant was added 6·0 ml. conc. H_2SO_4 and the mixture heated in a boiling water bath for 5 min. After cooling, estimations were carried out in a Unicam spectrophotometer at 322 mμ. After subtracting the value of the paper blank, the densities of both control and experimental samples were calculated from the equation for the standard regression line. Values were calculated per unit of tissue and the difference between the experimental and control values gave the amount of lactose synthesized per unit tissue. Experiments with pure lactose ('Analar') by this procedure gave a recovery of 94 ± 1·3%.

Estimation of deoxyribonucleic acid (DNA). A washed mince preparation of the mammary tissue was used. About 600 mg. of this material was homogenized in a Potter–Elvehjem (22) homogenizer with 1·0 ml. distilled H_2O, further H_2O being added to dilute the homogenate to contain about 200 mg. tissue per ml. The extraction of DNA was carried out by the method of Schneider (23), except that the phospholipid extraction was not carried out (Smith (8); Greenbaum & Slater (24,25)). The DNA was estimated by the method of Dische (26) at 605 mμ on a Unicam spectrophotometer using DNA prepared from herring sperm (Light & Co.) as a standard. The DNA was expressed per unit dry weight (DW) of this preparation.

Statistical methods. For all groups the means and the standard error of the means were calculated. Differences between groups were examined by the 't' test after testing the homogeneity of populations by the 'F' test. Where the populations of groups differ, significance of differences was tested using (n_1-1) degrees of freedom instead of (n_1+n_2-2) as recommended by Snedecor (27). To establish relationships between factors within the same stage of lactation, coefficients of correlation were calculated; and to study trends in factors in declining lactation regression coefficients were calculated. The significance of these were tested using Fisher's table of 't' (Fisher & Yates (28)).

RESULTS

Determination of lactation curve. Five animals were used, three of which were nursing their first litters, one her second and the remaining her third. The daily milk yields of the animals are shown in Fig. 1. It is clear from this figure that the peak of lactation occurs between days 2 and 3. The rate of decline varied considerably as between individuals, but the slope was not so steep as that described by Nelson *et al.* (16) for the guinea-pig. The average regression equation for the decline in yield was

$$y = 10{\cdot}65 - 0{\cdot}79x,$$

where y is the milk yield in grammes and x is the time in days.

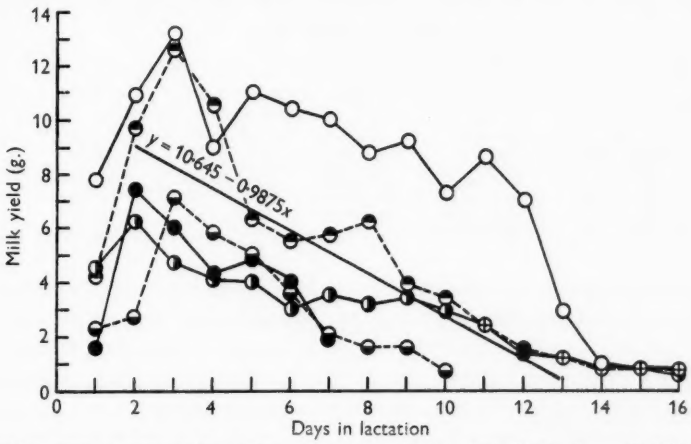

Fig. 1. Lactation curve of guinea-pigs. Animal No.: ○——○, I-1 (first litter); ●——●, I-3 (first litter); ◑——◑, I-5 (first litter); ◒---◒, II-2 (second litter); ◓---◓, III-4 (third litter); ——, standardized regression line during decline.

Estimation of rate of lactose synthesis at various stages of lactation. Inspection of the lactation curve shown in Fig. 1 suggested that groups of animals should be investigated at day 0 (i.e. day of parturition), days 2–3 (at peak of lactation), day 8 (during declining phase of lactation) and day 15 (just prior to weaning). Accordingly, five animals were investigated at each of these stages of lactation. With the exception of one killed on day 0 and one on day 3, all were undergoing their first lactation. Details of live weight and mammary gland weights for these groups, together with the milk yields of those killed at the peak of lactation, are given in Table 1. The mean peak milk yield for this group of

animals (11·0 ± 0·78 g./day) is not significantly different from that of the five animals (8·8 ± 1·23 g./day) used in the preliminary lactation curve experiment. The weights of the mammary glands tended to be highest at the peak of lactation, but Table 3 shows that the difference between stages of lactation were not significant.

Table 1. *Mean weights of animals and their mammary glands at various stages of lactation*

(Means of five animals with S.E.)

Stage (day of lactation)	No. of animals	Live weight (g.)	Weight of whole mammary gland (g.)	Milk yield (g.)
I (0)	5	787·2 ± 46·26	8·9 ± 2·15	—
II (2 or 3)	5	764·6 ± 42·53	15·1 ± 2·21	11·0 ± 0·78
III (8)	5	683·9 ± 25·67	11·9 ± 0·61	—
IV (15)	5	651·1 ± 84·89	10·9 ± 1·14	—

Details of dry matter, DNA and lactose content, oxygen consumption and lactose synthesis of the mammary tissue are given in Table 2. Inspection of the table shows that considerable amounts of lactose were synthesized *in vitro* at all stages of lactation. When calculated per mg. DNA the values for lactose synthesis show a tendency to increase towards the peak of lactation, then fall away.

Table 2. *Effect of stage of lactation on oxygen consumption and lactose synthesis rate of mammary gland slices*

(Means of observations on five animals with S.E.)

			Oxygen consumption		Lactose synthesis	
Stage	% DM of tissue slices	DNA μg./ mg. DM	μl. O_2/ mg. DM/hr.	μl. O_2/ mg. DNA/hr.	mg. lactose/ g. DM/hr.	mg. lactose/ g. DNA/hr.
I	19·0 ± 0·20	43·7 ± 2·23	5·7 ± 0·23	131·7 ± 10·71	40·2 ± 1·56	934·7 ± 75·64
II	19·5 ± 0·33	30·1 ± 0·65	6·8 ± 0·37	226·2 ± 12·22	35·1 ± 1·34	1162·8 ± 33·66
III	19·7 ± 0·44	30·6 ± 1·73	6·2 ± 0·26	203·8 ± 6·55	26·5 ± 1·37	872·2 ± 42·61
IV	17·6 ± 0·15	36·9 ± 0·91	7·0 ± 0·04	191·1 ± 4·63	20·3 ± 0·47	551·7 ± 15·54

The dry-matter content of the slices was remarkably constant, being about 19% for all stages, except at weaning when it fell to 17%. This difference between the last and earlier stages being statistically significant ($P = 0·01$ or less, see Tables 2 and 3). The DNA content of the dry matter was highest at parturition, lowest during the peak and the decline of lactation, but rising again at the time of weaning (see Tables 2 and 3). Oxygen consumption on a dry-weight basis was lowest at parturition and highest at time of weaning, on a DNA basis it was lowest at parturition but highest at the peak of lactation (Tables 2 and 3). Lactose synthesis on the dry-weight basis was lowest at time of weaning and highest at parturition, on a DNA basis it was lowest at time of weaning but highest at peak of lactation (Tables 2 and 3).

DISCUSSION

This work must obviously be regarded as a preliminary step in the study of the relation between lactational performance and milk synthesis rate owing to the small number of animals involved, the fact that milk yields were not recorded for animals in the main experiment at stages other than peak of lactation, and to the fact that DNA was not estimated in fresh unwashed tissue as well as in the minced and sliced preparations to

Table 3. *Significance of differences between various stages*

Item	Between stages I–II	I–III	I–IV	II–III	II–IV	III–IV
Live weight	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Weight of fresh mammary gland	N.S.	N.S.†	N.S.	N.S.†	N.S.	N.S.
% of dry-matter of mammary slices	N.S.	N.S.	****	N.S.	****	**†
DNA (DW)	***†	***	*	N.S.†	****	**
O_2 (DW)	*	N.S.	***†	N.S.	N.S.†	*†
O_2 (DNA)	****	****	****	N.S.	*	N.S.
Lactose (DW)	*	****	****†	***	****†	**†
Lactose (DNA)	*	N.S.	***†	****	****	***†

Remarks: N.S., not significant; *, significant ($0{\cdot}02 < P < 0{\cdot}05$); **, significant ($0{\cdot}01 < P < 0{\cdot}02$); ***, significant ($0{\cdot}001 < P < 0{\cdot}01$); ****, significant ($P < 0{\cdot}001$).

† Indication of different population (D.F. is 4). The cases without this mark show two populations belonging to the same, and D.F. is 8.)

Table 4. *Coefficients of correlation*

Combination	Coefficients		Combination at stage II (peak period)	Coefficients
O_2: lactose (DW)				
Stage I	+0·929*		Live weight	+0·313 N.S.
II	+0·872 N.S.		% of dry matter of mammary slices	−0·486 N.S.
III	+0·913*		DNA (DW)	+0·414 N.S.
IV	+0·375 N.S.	Milk yield	O_2 (DW)	+0·869 N.S.
O_2: lactose (DNA)				
Stage I	+0·982***		O_2 (DNA)	+0·730 N.S.
II	+0·876 N.S.		Lactose (DW)	+0·939**
III	+0·928*		Lactose (DNA)	+0·955**
IV	−0·447 N.S.			

Remarks: N.S., not significant; *, significant ($0{\cdot}0{\cdot}2 < P < 0{\cdot}05$); **, significant ($0{\cdot}01 < P < 0{\cdot}02$); ***, significant ($0{\cdot}001 < P < 0{\cdot}01$); ****, significant ($P < 0{\cdot}001$).

enable DNA content and lactose synthesis *in vitro* to be calculated on a whole gland basis.

Although the demonstration of lactose synthesis *in vitro* is not a new observation, most workers have been content to demonstrate that it does occur in the fully lactating gland. Comparison of the rates of lactose synthesis observed in the present study with the scanty data quoted by previous workers indicates that the present values are considerably higher. The reason for this difference is as yet undetermined. The present investigation shows that per unit DNA there is a progressive increase of lactose synthesis to the peak of lactation and then a pronounced drop. If it be assumed that the DNA content of the tissue is proportional to the number of cells present (for discussion see Greenbaum & Slater(24,25)), then the present results indicate that the functional activity of the individual alveolar cells as judged from the *in vitro* O_2 consumption and lactose synthesis rates is maximal at the peak of lactation. While a high correlation (see Table 4) was found between lactose synthesis rates and oxygen consumption rates at the onset and decline of lactation, the correlation was lower at the other stages which suggests that changes occur in the character of the cellular metabolism during lactation. The synthetic activity is high at the beginning of lactation, but declines as the cells age despite the continuation of the suckling stimulus, whereas the respiratory activity tends to remain at a high level throughout.

The high correlation (see Table 4) between milk yield at the peak of lactation and the *in vitro* lactose synthesis rates both on DW and DNA bases is interesting, but further studies are necessary to determine the true significance of this relationship. (22) (23) (24)

The results, however, show promise that the estimation of lactose synthesis *in vitro* may be a reliable guide to the synthetic activity of the tissue *in vivo* and consequent milk production. (25) (26) (27) (28)

SUMMARY

The shape of the lactation curve in guinea-pigs has been studied. The peak was found to occur between days 2 and 3.

The oxygen consumption and lactose synthesis rates were investigated in mammary gland slices and results expressed on both DNA and DW bases.

Oxygen consumption rates on a DNA basis tended to be low at the onset of lactation and high at the peak. Lactose synthesis on a DW basis tended to decline linearly from the onset of lactation, but on a DNA basis showed a peak coinciding with that of the lactation curve.

The significance of these findings has been discussed.

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692. THE INFLUENCE OF CARBON DIOXIDE ON THE GROWTH OF LACTIC STREPTOCOCCI

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(With 3 Figures)

It has long been known that the presence of carbon dioxide in the medium is essential for the growth of many species of bacteria. One of the earliest observed examples is that of *Brucella abortus* (Huddleston (1)) which grows readily only in an atmosphere containing 10% of CO_2. Valley & Rettger (2) found that the need for CO_2 was shared by a large number of species when they were grown on solid media. Briggs (3) observed that lactobacilli gave improved growth on the surface of agar in an atmosphere containing 10% of CO_2. Gladstone, Fildes & Richardson (4) investigated the effect of CO_2 on a variety of organisms growing in liquid media of simple composition, and found that the CO_2 requirements of some species was modified by changes in composition of medium.

Carbon dioxide is needed by many organisms to initiate growth. Usually where a bacterium produces CO_2 during growth its initial requirements are provided for by the CO_2 present in the inoculum added to the fresh batch of medium. Thereafter, the organism supplies its requirements by its own production of CO_2. But where an organism produces little or no CO_2 and circumstances are such that the gas is absent or is continuously removed from the medium, the organism may either be prevented from growing at all or be severely restricted in its growth, depending on the composition of the medium.

The homofermentative lactic streptococci produce very little CO_2 during growth, but since pasteurized milk, in which they are commonly grown during dairy manufacturing operations, normally contains about 3% by volume, their CO_2 requirements for active growth are usually satisfied. There do occur circumstances, however, particularly under laboratory conditions, where their rate of growth may be reduced by reason of a sub-optimal concentration of CO_2 in the medium. Our attention was drawn to this possibility by the observation that strains of *Streptococcus lactis* and *Str. cremoris* growing in sterilized skim-milk showed a delayed acid production when the culture tubes were inverted at half-hour intervals instead of being left undisturbed. The delay was mainly due to a longer lag period in the inverted cultures, the rate of acid production later in the experiment being similar to that in uninverted cultures.

Continuous agitation of a skim-milk culture by means of a stream of air gave a greater delay in acid production than did periodic inversion. Agitation by means of a stream of nitrogen gave still greater delay. The admixture of some CO_2 with the nitrogen resulted in a higher initial rate of acid production similar to that in an undisturbed culture. It was thus evident that lactic streptococci required some CO_2 in solution for optimal growth in the milk medium. The work to be described was carried out in an attempt to clarify the position.

METHODS

The cultures used were all stock strains of *Str. lactis* and *Str. cremoris* which have been maintained in this laboratory in daily subculture in skim-milk for a number of years. They had been plated out on agar at frequent intervals and re-isolated to maintain their acid-producing activity.

For investigation of the effect of the passage of different gases and gas mixtures on the growth of the streptococci a batch of skim-milk was sterilized in the autoclave (15 lb./sq.in. for 15 min.). The milk was then inoculated with 2% of a 24 hr. (22° C.) skim-milk culture of a streptococcal strain, and after a thorough mixing the inoculated milk was distributed aseptically in 4·5 ml. quantities into a series of sterile $6 \times \frac{3}{4}$ in. test-tubes closed with Astell stoppers. The tubes were incubated in a water-bath at $30 \pm 0{\cdot}5$ °C.

Where a culture was to have gas bubbled through it during incubation the Astell stopper was replaced with a stopper fitted with inlet and outlet tubes, the former drawn out to a diameter of approximately 2 mm. and reaching to the bottom of the culture. The gas-tube system was wrapped and sterilized prior to the experiment and fitted aseptically to the culture tube. The inlet tubes from a series of test cultures were connected by very short lengths of rubber tubing to branches of a manifold, and a screw clip on each branch made it possible to equalize the gas flow through the cultures. Mixtures of gases (e.g. N_2 and CO_2) were produced by metering the individual gases from cylinders through Greiner flow meters into the manifold. Where air was the gas to be used the manifold system was connected to the outlet tubes from the cultures, and a vacuum pump attached to the manifold was used to aspirate air through the cultures. In all experiments the rate of gas passage was of the order of 30 ml. per minute.

The 4·5 ml. cultures were assumed to be exact replicates, and the smoothness of the acid-production curves obtained bore out this assumption. For determination of acid production in the course of an experiment a culture, detached from the system at appropriate time intervals, was titrated with NaOH to the phenolphthalein end-point and then discarded. Examination of samples under the microscope and plate counts in some experiments showed that increase in acidity under all the experimental conditions used was directly proportional to the increase in bacterial numbers.

Estimation of the CO_2 content of the laboratory air was carried out by aspiration of a large measured volume (about 20 l.) at a slow rate through a Pettenkofer tube containing standard baryta solution. The baryta solution was back-titrated with oxalic acid to the phenolphthalein end-point. The CO_2 content of the milk medium and of milk cultures grown overnight was determined by aspiration of CO_2-free air through 200 ml. of the milk or culture, and absorption of the entrained CO_2 in standard baryta. Trials showed that aspiration for 1 hr. with the milk or culture warmed to 30° C. was sufficient to remove all CO_2. Acidification of milk was not necessary under the conditions used. With 200 ml. quantities the method gave results significant to 0·1% CO_2 by volume.

RESULTS

Eleven strains of lactic streptococci were investigated, three of *Str. lactis* and eight of *Str. cremoris*. All strains of both species showed a need for the presence of CO_2 in the medium, and in both species there were strains with a very low requirement and others

with a rather higher requirement for CO_2. To conserve space the results obtained with only two strains will be given in detail; they typify the general results. Figs. 1 and 2 illustrate the responses obtained with a low CO_2-requirement lactis strain ML_3 and the somewhat higher CO_2-requirement cremoris strain HP.

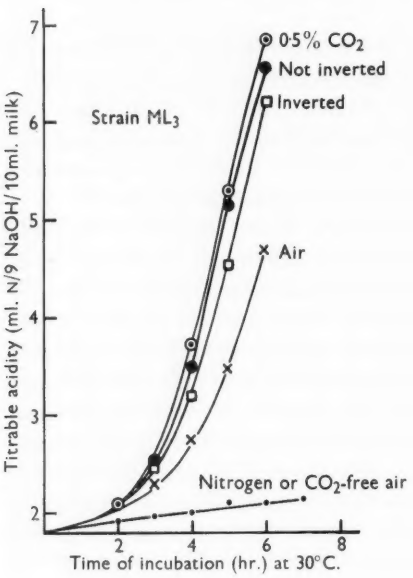

Fig. 1

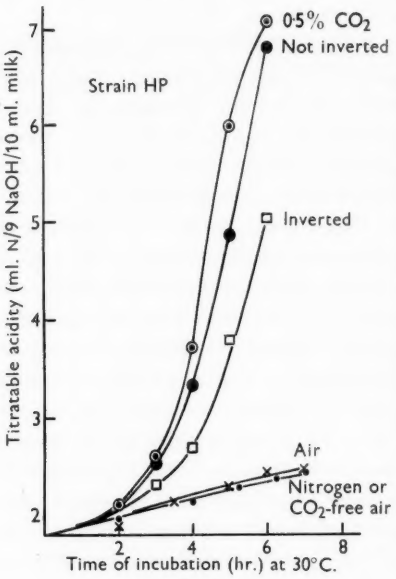

Fig. 2

Effect of inversion. It is evident from the curves that cultures of both strains, when inverted at half-hour intervals, showed a longer lag period and consequently a delayed acid production as compared with uninverted controls, the difference being greater with HP than with ML_3. With ML_3 the difference was small but it was invariably present. It seemed possible that this phenomenon might be linked with the tendency of long-chained streptococci to sink to the bottom of an undisturbed liquid culture. It could be postulated that if left undisturbed the bacteria would produce a favourable CO_2 concentration in their immediate vicinity, and that redistribution of the organisms throughout the culture as a result of inversion would render their environment less favourable. At 30° C. strain HP formed the longest chains of any of the cultures used and ML_3 formed mainly diplococci. Examination under the microscope of samples taken from different depths in the culture tube showed that HP did in fact tend to sink gradually in an undisturbed culture, whereas ML_3 showed a more even distribution throughout the milk. The other cultures falling between these two in chain length gave intermediate results in difference of acid production between inverted and non-inverted cultures. It thus appeared that there was a connexion between the tendency of long-chained strains to settle in an undisturbed culture and the effect of inversion on the rate of acid production, and that a CO_2 effect might be involved. This point will be discussed later.

Effect of passage of CO_2-free gases. The passage of nitrogen or of CO_2-free air through cultures during incubation had the same effect on all strains. It will be seen from the

curves that under these conditions acid production followed a straight line of very slight slope. Examination under the microscope and plate counts showed that there was a slow multiplication of the organisms; the acid was not produced entirely by non-proliferating organisms of the original inoculum. Absence of free CO_2 did not therefore inhibit growth completely. In one experiment where a culture was incubated overnight with continuous passage of nitrogen, the milk culture coagulated at some time between 12 and 24 hr. from the time of inoculation. The apparently straight line must, therefore, represent a very prolonged lag phase from which the organisms finally emerge even in the absence of CO_2.

Effect of traces of CO_2. Passage of air (drawn from the laboratory) through cultures gave an effect which varied with the strain under investigation. Figs. 1 and 2 show the two types of result. With all strains there was a lower initial rate of acid production than in the uninverted control. With HP and four other strains the curve was only a little above that for passage of a gas containing no CO_2. With ML_3 and five other strains the curve was intermediate between the control and nitrogen curves, and its exact position varied somewhat among the six strains which reacted in this way. The laboratory air was estimated to contain approximately 0·04% CO_2 by volume (Dagley & Hinshelwood (5) give a value of 0·03% for the concentration of CO_2 in fresh air). The results indicate, therefore, that the equilibrium concentration of CO_2 produced in the milk by the passage of gas containing this small proportion of CO_2, stimulates growth of some of the strains of lactic streptococci, but not to such an extent that the lag phase is reduced to a minimum.

Effect of larger proportions of CO_2. Passage of nitrogen containing 0·5% of CO_2 through cultures gave an initial rate of acid production equal to that in an uninverted control with ML_3 and similar cultures, and rather greater than that in the control with HP and similar cultures which evidently have a somewhat higher CO_2 requirement. Increase in the CO_2 proportion to more than 0·5% did not have any further effect. It was not possible with the metering device available to produce artificial mixtures of gases containing less than 0·5% CO_2 with accuracy, but in one experiment with HP it was found that about 0·1% CO_2 in nitrogen gave acid development in a culture very similar to that which occurred in an uninverted control. It must of course be remembered that CO_2 concentration in an uninverted control was not necessarily optimal for cultures with the higher CO_2 requirement.

The results thus indicated that a minimal lag period occurred with the various strains of streptococci when the gas mixture passed through the skim-milk medium contained concentrations of CO_2 falling within the range 0·04–0·5%.

Carbon dioxide content of skim-milk cultures

The results given above are all in terms of the proportion of CO_2 present in the gas passed through the milk cultures. It was desirable, however, to know the actual CO_2 content of the milks in the various experiments, because only then could a comparison be drawn between the CO_2 effect in undisturbed cultures and those through which gas was passed. The CO_2 content of milk after various treatments was determined by aspirating CO_2-free air through 200 ml. of the milk in a flask, trapping the CO_2 in baryta and back-titrating as described in the section on methods. The use of 200 ml. for the estimation limited the accuracy to 0·1% by volume.

The raw milk from which the experimental skim-milk was prepared gave values over a 7-day period ranging from 2·9 to 4·7% CO_2. The skim-milk after sterilization in the

autoclave gave values ranging from 0·3 to 0·5% CO_2. Passage of laboratory air containing 0·04% CO_2 through the sterile skim-milk for 2 hr. lowered the CO_2 content to an equilibrium value of 0·2%. Passage of nitrogen containing 0·5% CO_2 for 2 hr. produced an equilibrium value of 2·3%.

It appears, therefore, that the minimum CO_2 content for optimal initial growth (minimum lag period) of lactic streptococci lies between 0·2 and 2·3%. None of the strains gave optimal growth with 0·2% CO_2 present, but in all probability the requirements of several strains like ML_3 would be satisfied at a figure not much higher than this. There were indications from one experiment that even strains like HP with rather higher CO_2 requirements might give optimal growth at levels considerably below 2·3%. For these latter strains, however, it is evident that the 0·3–0·5% CO_2 found in sterilized milk is not sufficient to support optimal initial growth, and that therefore they may often show a prolonged lag phase under laboratory conditions.

Formation of CO_2 *by lactic streptococci*

It was of interest to determine whether the streptococci were capable of producing a significant amount of CO_2 during their growth in skim-milk, and thus supplying part or all of their requirements for the gas, except in the initial stages of growth in a new medium or when CO_2 was swept away by a gas stream. 24 hr. skim-milk cultures (30° C.) of all the streptococci under investigation were tested for the production of CO_2. Carbon-dioxide-free air was aspirated through 200 ml. quantities of clotted culture and the entrained CO_2 estimated as previously described. The CO_2 content of an uninoculated control milk was also estimated and the result subtracted from the total CO_2 content of each clotted culture. All the strains of *Str. lactis* and *Str. cremoris* produced CO_2 during growth, the increases ranging from 0·5 to 1·3%. This formation of CO_2 by the homofermentation streptococci is in accord with expectation, since it is known that these organisms form traces of diacetyl which necessarily involves the coincident formation of CO_2.

It would appear that in an undisturbed milk culture a suboptimal concentration of CO_2 will only delay initial growth of the lactic streptococci and prolong the lag period. When growth is established, as it eventually is even in the initial absence of extracellular CO_2, the bacteria probably supply their own requirements.

Substitution of other substances for CO_2

It has been found with several types of bacterium that active growth will occur in the absence of CO_2 provided that certain other substances are present. Ajl & Werkman (6) showed that glutamic acid as well as certain other C_4 and C_5 dicarboxylic acids would replace CO_2 in the medium for the growth of *Bacterium coli* and *B. aerogenes*, probably because these compounds are necessary in the Krebs cycle and, if absent, have to be formed by reaction of CO_2 with pyruvate. Gerhardt & Wilson (7) found that in the case of *B. abortus* glutamic acid could not replace CO_2. Tuttle & Scherp (8) found with meningococci that yeast extract could substitute for CO_2. They suggested that it acted by stimulating production of CO_2 by the organism rather than by replacing CO_2 in the metabolism.

The effect of an addition of yeast extract (Oxoid bacteriological) was tested in skim-milk cultures of the lactic streptococci with continuous passage of nitrogen during incubation. All the strains responded to the extract, giving growth and acid production equal to that in the uninverted control when a certain minimum (about 0·4%) of yeast extract was

present. The response of the various strains to yeast extract was not linked to their different levels of CO_2 requirement. Fig. 3 shows the results obtained with ML_3. It is evident that with continuous passage of nitrogen the presence of yeast extract restored the activity of the streptococci until, at a level of 0·5%, it was equal to that in a sterile skim-milk containing the normal amount of CO_2 (0·3–0·5%). An increase in proportion of yeast extract beyond 0·5% had no further effect. The uninverted culture containing 0·5% yeast extract was more active than the control without yeast extract. The reason for this is not clear.

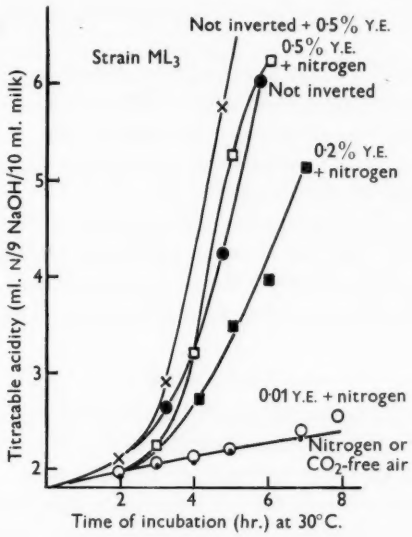

Fig. 3

The exact roles of CO_2, yeast extract, and regular inversion are difficult to define, but the fact that HP, a strain with a somewhat higher CO_2 requirement, reacted similarly to ML_3 to the presence of a suboptimal proportion of yeast extract such as 0·2%, suggests that yeast extract acts by substituting for CO_2 in the bacterial metabolism, rather than by stimulating CO_2 production by the bacterium. This is by no means proven, however, especially since it was found that in undisturbed cultures the presence of yeast extract did result in an increase in CO_2 production by the streptococci.

Mechanical disturbance of a culture

Although the original observation on the effect of periodic inversion of a culture led to the finding that CO_2 plays a part in controlling the initial rate of growth of lactic streptococci, no direct connexion between the two phenomena had been established in the course of the experiments.

There was the possibility that incorporation of air in the culture milk as a result of inversion interfered in some way with the growth of the streptococci. On the other hand, there was a possibility that inversion caused a reduction in the concentration of CO_2 round any streptococci which had sunk to the bottom of an undisturbed culture, and that consequently the lag period was prolonged.

The first alternative was tested by covering a culture with liquid paraffin and keeping the milk in continuous movement by means of a mechanically operated pipette, which alternately drew up and ejected some of the culture without breaking the paraffin seal. Such a culture gave results similar to those obtained from a periodically inverted culture. Hence, it was concluded that incorporation of air was not the factor responsible for prolongation of the lag period.

The second alternative was tested by observing the effect of periodic inversion on a skim-milk culture.

(*a*) containing sufficient yeast extract to give optimum growth in the absence of CO_2, and

(*b*) through which 1% CO_2 in nitrogen had been passed for 2 hr. before inoculation.

Uninverted controls were run in each instance. The results showed that inversion had no significant effect when yeast extract was present, but that the usual difference observed as a result of inversion occurred in the cultures containing an adequate proportion of CO_2 at the start of the experiment.

It would appear, therefore, that the hypothesis that inversion prolongs the lag period by dispersion through the medium of an accumulation of CO_2 round the sedimented streptococci is untenable. The reason for the effect of mechanical disturbance of a culture on the initial growth rate of some strains of lactic streptococci in plain skim milk remains obscure.

DISCUSSION

Although it is clear from the results detailed above that the homofermentative lactic streptococci require a small concentration of CO_2 in a milk medium for optimum initial growth, it would appear that they are unlikely to suffer from a shortage of CO_2 in the milk used in commercial dairy manufacturing processes. The position may be different in the laboratory where the process of sterilization removes most of the CO_2 normally present in milk. For some strains of streptococci, sterilized skim-milk is deficient in CO_2, and in consequence they show a prolonged lag phase, thus giving low acidity values during the first few hours of growth. Little or no difference would be observed, however, between cultures deficient in and those amply supplied with CO_2 after overnight incubation. The results indicate that where the acid-producing activity of lactic streptococci in milk over a period of 4–6 hr. is under investigation, it is advisable to ensure that there is enough CO_2 in the medium or to have present an additive such as yeast extract which can substitute for CO_2.

SUMMARY

The initial rate of growth of *Str. lactis* and *Str. cremoris* in skim-milk depends upon the proportion of CO_2 present in solution. When CO_2 is constantly swept from the milk medium by a stream of CO_2-free gas all strains show a very prolonged lag period. They eventually emerge from this lag period after more than 8 hr. and grow well enough to coagulate the milk within 24 hr. For optimal initial growth the lactic streptococci require the presence in solution in the milk of proportions of CO_2 within the range 0·2 and 2·3% by volume. Some strains have a higher CO_2 requirement within this range than others. Skim-milk after sterilization contains less than the optimum proportion of CO_2 for many strains of lactic streptococci.

Yeast extract in a proportion of about 0·5% can substitute for CO_2 in skim-milk.

The periodic inversion of cultures in plain skim-milk reduces the initial rate of growth of some of the streptococci, particularly those which form long chains. The reason for this is unknown. It does not occur when yeast extract is present.

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693. A REJECTION TEST FOR RAW MILK

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(With 11 Figures)

Milk may be rejected for several reasons. The most usual cause is taint. This can be due to feed or to an upset in the metabolism of the cow, or the taint may be caused by the action of micro-organisms. Further, the appearance of the milk (it may contain blood or visible dirt) or its chemical composition, may make it unacceptable.

We are concerned here with estimating the activity of micro-organisms in milk. If this can be measured accurately it will assist in difficult decisions of acceptance and in the alteration of the standard of acceptance according to the purpose for which the milk is to be used.

The methods hitherto used to determine microbiological activity in milk have been discussed in a review (1) and the merits of the various tests need not be further considered here. The most widely used rejection test in this country during the past 15 years has been the 10 min. resazurin test. This test, although without doubt the most useful rejection test now used, suffers from certain defects. In some cases milks of moderately good keeping quality are rejected because of the presence of a large number of bacteria which have no effect on keeping quality, but which reduce the dye, resazurin. Garvie & Rowlands (2) have shown that the micro-organisms which are dominant at the end-point of keeping quality (the clot-on-boiling point), after the milk has been stored under normal atmospheric conditions, are the group N (lactic) streptococci. A test for so-called 'lactic acid' which has been widely used in the past is the titration with N/9 sodium hydroxide to the phenolphthalein end-point. While this test measured total acidity from all sources, i.e. natural acidity from casein and developed lactic acid, if any, it was also influenced by the buffering capacity (i.e. the chemical composition) of the milk. What is required is a test which will measure the *developed* lactic acid in milk, which is unaffected by the composition of the milk.

A rapid method for the determination of lactic acid by the formation of complex ferric lactate with ferric chloride has been described by Ling (3). The present work reports an examination of this method with a view to adopting it as a rejection test for raw milk.

EXPERIMENTAL

Reagents

The reagents used by Ling (3) for the determination of lactic acid in milk which have been found satisfactory by Karamizrak (4) in this laboratory are: barium chloride crystals, 98·75 g./l.; sodium hydroxide, 0·66N; zinc sulphate crystals, 225 g./l.; 5% ferric chloride crystals (hexahydrate), 5 g. plus 12·5 ml. N hydrochloric acid/100 ml. (a stock solution of this is kept in the refrigerator); 1% ferric chloride (5% ferric chloride solution diluted five times immediately before use).

Examination of methods used in the test

Filtration

Ling suggested the following method for precipitation of the proteins from 25 ml. of milk. 10 ml. of barium chloride, 10 ml. of sodium hydroxide and 5 ml. of zinc sulphate solutions were added from burettes with gentle shaking after the addition of the first two reagents, and vigorous shaking after the addition of the zinc sulphate solution before filtration. We have found that the rapid addition of reagents from automatic pipettes with only a slight mixing of liquid between each addition is satisfactory providing vigorous shaking before filtration is given.

Quantity of reagents

Ling noticed that with fresh raw milk, the intensity of the yellow coloration increased with additional quantities of ferric chloride. He found that the ferric chloride had not completely reacted. When small volumes (0·25 ml.) of 1% ferric chloride solution were added to fresh raw milk the reaction was not complete, and the addition of lactic acid (0·005%) increased the colour. For the preparation of his standard curves he therefore used the minimum quantity of ferric chloride which would react completely with the lactic acid present. For ordinary samples he recommended that the quantity of ferric chloride should be obtained from an approximate determination of lactic acid, using the ferric-salicylate-ferrocyanide reagent.

To determine the effects of varying quantities of reagent on filtrates of fresh raw milk, and also to check the quantities of 1% ferric chloride solution required to react with varying quantities of lactic acid, the following experiments were made.

(i) Varying quantities of 1% ferric chloride solution were added to 10 ml. aliquots of standard aqueous lithium lactate solutions. Readings were made on a Spekker absorptiometer using a 2 cm. cell, a drum setting at 1·00 with water, and violet filters (Ilford 601), which had been shown to be most suitable by both Ling and Karamizrak. The results obtained have been plotted in Fig. 1. The amount of ferric chloride required to react with varying quantities of lactic acid, and the fact that excess reagent does not interfere in the colour development, can be clearly seen.

(ii) Varying quantities of 1% ferric chloride solution were added to 10 ml. aliquots of filtrate from two samples of fresh raw milk. Readings were made on a Spekker absorptiometer as before, but a 1 cm. cell was used instead of a 2 cm. cell.

The results in Table 1 show that the yellow colour obtained with raw milk is only partly due to the colour of the reagent, and that the increasing colour obtained with increasing quantities of ferric chloride reaches a maximum in fresh raw milk when 2 ml. of 1% ferric chloride have been added to 10 ml. of milk filtrate.

(iii) A similar experiment was carried out by adding varying quantities of 1% ferric chloride to filtrates obtained from milk to which lithium lactate had been added in concentrations equivalent to 0·01–0·02% lactic acid. The results obtained are shown in Fig. 2. Curves 1–9 were obtained from milks to which was added 0·01, 0·02, 0·04, 0·06, 0·08, 0·10, 0·12, 0·16 and 0·20% lactic acid, respectively. As it is evident that 2 ml. of ferric chloride was sufficient to react completely with lactic acid originally in the milk plus 0·20% developed lactic acid in the milk, this volume was chosen as a satisfactory quantity for routine use. This would be sufficient for all concentrations of lactic acid likely to be present in milk filtrate up to the clot-on-boiling (C.O.B.) end-point.

For accurate determination of the lactic acid Ling suggested that sufficient ferric chloride should be added to react with lactic acid present, with only a 'minimum blank'. For the present purpose, however, it is essential to add the same quantity of ferric chloride to all samples. Although 2 ml. of 1% ferric chloride was found to be sufficient,

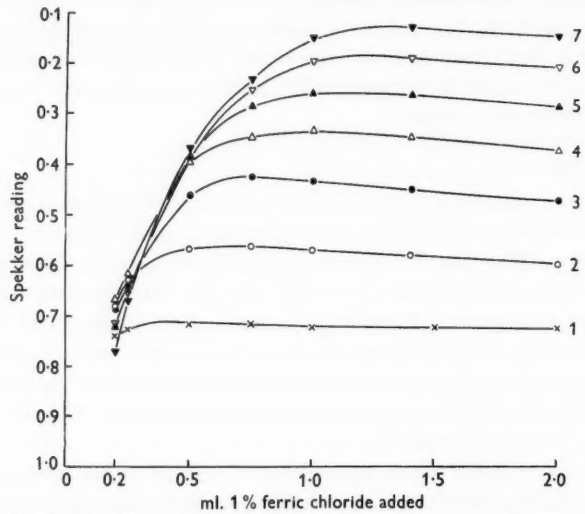

Fig. 1. Amounts of ferric chloride required for complete reaction with varying quantities of lactic acid. (All readings made on Spekker using 2 cm. cells.) Key to strengths of lactic acid solutions: 1, 0·005%; 2, 0·01%; 3, 0·02%; 4, 0·03%; 5, 0·04%; 6, 0·05%; 7, 0·06%.

Table 1. *The effect of varying quantities of ferric chloride on the colour developed in fresh raw milk*

Volume of $FeCl_3$ added (ml.)	Drum reading (1 cm. cell)				
		Sample 1		Sample 2	
	Water	Reading	Difference between sample and water	Reading	Difference between sample and water
0·1	0·996	0·960	0·036	0·960	0·036
0·15	0·995	0·951	0·041	0·950	0·045
0·25	0·995	0·942	0·053	0·937	0·058
0·5	0·989	0·912	0·077	0·911	0·078
0·75	0·981	0·886	0·095	0·893	0·088
1·0	0·974	0·870	0·104	0·880	0·094
1·5	0·961	0·847	0·114	0·858	0·103
2·0	0·954	0·835	0·119	0·843	0·111
2·5	0·943	0·823	0·120	0·833	0·110
3·0	0·936	0·815	0·121	0·825	0·111
3·5	0·926	0·811	0·115	0·819	0·107

this amount gave a large blank in fresh raw milk, see Table 1. However, it was decided that this did not matter for the present purpose of the test, as it was only necessary to determine the residual life of a milk rather than the actual lactic-acid content. Further, results using this technique cannot be regarded as values of lactic acid in the milk even if a blank lactose and reagent is subtracted, as it is not possible to estimate the amount of

interfering materials which probably vary with the degree of ageing and from sample to sample. It was therefore decided that no blank should be subtracted, and that the results should be expressed as 'apparent lactic acid'.

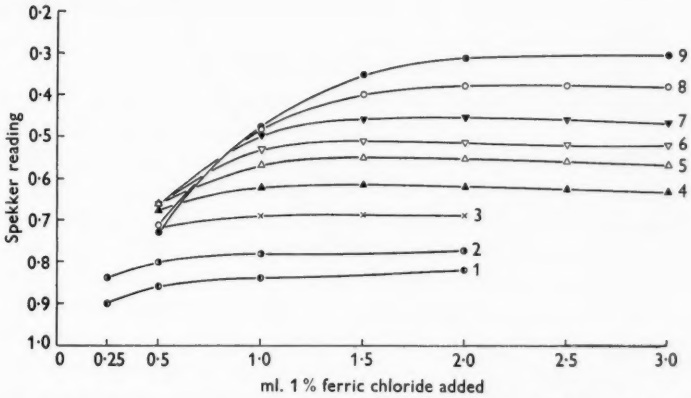

Fig. 2. The effect of adding varying quantities of 1% ferric chloride to filtrates from milk to which varying quantities of lithium lactate had been added. (All readings made on Spekker using 1 cm. cells.) Key to strengths of lithium lactate solutions as lactic acid: 1, 0·01%; 2, 0·02%; 3, 0·04%; 4, 0·06%; 5, 0·08%; 6, 0·10%; 7, 0·12%; 8, 0·16%; 9, 0·20%.

Preparation of standard lactic-acid curve

To determine the lactic-acid content of milk samples, it was necessary to prepare a standard curve from which to read the results. Ling used A.R. lactic acid as a standard, and stored the solutions at room temperature for 3 months. However, we found that a 10% solution was not completely hydrolysed after 3 months, and that lactides and/or lactyl lactic acid were present. This was evident when the solution was standardized by the method of Hickinbotham (5), and was clearly demonstrated by the reaction of the solution in its original state, and after complete hydrolysis, with ferric chloride. An aliquot of the 10% solution was diluted with distilled water to give a 1% solution. This was used for the preparation of a set of standard solutions from 0·01 to 0·1%. To 10 ml. of each of these standard solutions was added 2 ml. of 1% ferric chloride solution. As before, the yellow colour developed was measured with a Spekker absorptiometer, but with a 1 cm. cell. An equal volume of the 10% solution was then boiled with excess sodium hydroxide for 20 min. before the solution was diluted to 1%, from which a second set of standards of concentrations from 0·01 to 0·1% was prepared. After adjusting the pH to 6·8 the ferric lactate colour was developed and measured as before. When the two sets of values were plotted, i.e. Spekker reading against lactic-acid concentration, the curves obtained were very different.

A third set of standard solutions of the same concentration range was prepared from a solution of lithium lactate containing 1·066 g. lithium lactate/100 ml., which is equivalent to a 1·0% lactic-acid solution. The curve obtained by plotting Spekker readings against lactic-acid concentration agreed well with that obtained with completely hydrolysed lactic-acid solution. These findings are shown in Fig. 3.

The need for care in ensuring complete hydrolysis of the lactides, and the necessity of standardizing the solution makes the use of lactic acid as a standard unnecessarily tedious, and the use of a crystalline lactate is more convenient.

Fig. 3. Standard curves obtained from solutions of lithium lactate and lactic acid before and after hydrolysis with sodium hydroxide. Plain circles, lactic acid (solid line incompletely hydrolysed, broken line hydrolysed); full circles, lithium lactate.

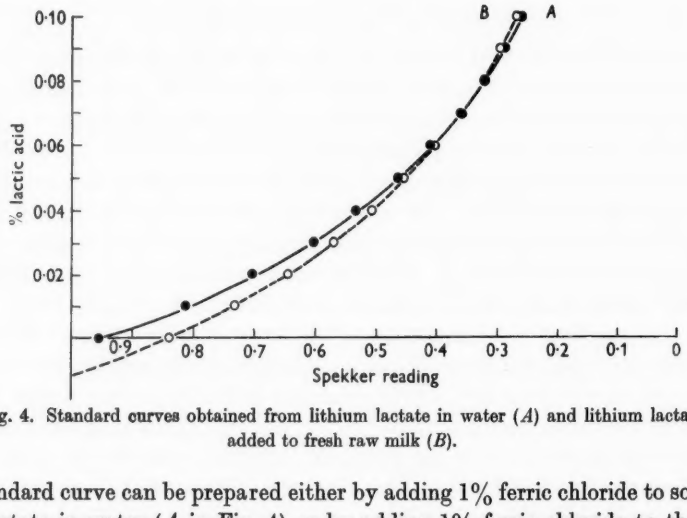

Fig. 4. Standard curves obtained from lithium lactate in water (*A*) and lithium lactate added to fresh raw milk (*B*).

The standard curve can be prepared either by adding 1% ferric chloride to solutions of lithium lactate in water (*A* in Fig. 4) or by adding 1% ferric chloride to the filtrates obtained from fresh milk samples to which varying quantities of lactic acid standard had been added before precipitation of the casein (*B* in Fig. 4).

The blank in *B* is much higher than *A* because of the colour of the milk filtrate with ferric chloride, see Table 1. In calculating the percentage recovery of lactic acid added to the milk, the difference between the blanks was subtracted from the values plotted in *B*. and the resulting values used to calculate the percentage lactic acid recovered, readings

being made from *A*. The acid recovered ranged from 84·3% with an addition of 0·01% lactic acid to 87·9% with 0·08% added.

The initial lactic-acid content of fresh milk is known to vary (3,6,7,10), and the recovery of lactic acid is only 80–90% (3). It was therefore decided that a solution of lithium lactate in water was a more reliable standard.

Method finally adopted

As a result of these investigations the method adopted for the determination of apparent lactic acid was as follows.

25 ml. of milk was pipetted into a stoppered 150 ml. flask to which was added 10 ml. of barium chloride, 10 ml. of sodium hydroxide and 5 ml. of zinc sulphate from automatic pipettes. The solutions were added quickly with only slight mixing between each addition. The flask was stoppered and shaken vigorously and the mixture was filtered through a fluted Whatman 12·5 cm., no. 40 filter-paper. To 10 ml. of the clear filtrate was added 2 ml. of 1% ferric chloride solution, and the colour measured on a Spekker absorptiometer using a 1 cm. cell, and violet filters (Ilford 601). The drum was set at 1·00 with water. Apparent lactic-acid concentrations in the milk filtrate were obtained by reading from the standard curve (Fig. 3). The apparent lactic acid in the milk can be considered as twice that in the milk filtrate. No allowance has been made for the volume of the precipitate as, for the accuracy required, correction is unnecessary.

RESULTS

Lactic acid in fresh raw milk

Before investigating the concentrations of apparent lactic acid developed in milk samples during ageing, it was considered desirable to establish the value of the apparent lactic acid in fresh raw milk directly after milking, and to find the variability between samples. Milk samples were taken from the cans after cooling at farms directly after morning milking, and were placed in sterile 4 oz. bottles. These samples were placed on ice and kept cool until tested 4–5 hr. after milking. The results of a selection of these samples are shown in Table 2. Samples marked with an asterisk were individual cow samples, and samples bracketed together were consecutive samples from the same cow; the remainder were mixed herd samples.

The values of lactic acid obtained agreed closely with the values given in the literature (3,6,7,8).

Table 2. *Estimation of lactic acid in fresh raw milk.* (*By Davidson's* p-*hydroxy diphenyl method*)

Meal	% lactic acid	Meal	% lactic acid
M	0·0027	E	0·0016
M	0·0020	M	0·0026
M	0·0027	E	0·0013
M	0·004	M	0·0013
E	0·0027*	E	0·0033
E	0·0032*	M	0·0029
E	0·0025*		
E	0·0012		
M	0·0010		

M, morning milk. E, evening milk.

Relationship between apparent lactic-acid concentration and keeping quality of milk

To determine whether a relationship exists between the keeping quality of milk and its apparent lactic-acid concentration, samples of morning milk were examined during the winter and summer periods. Samples were taken from cans on arrival at local creameries into sterile bottles and were brought to the laboratory within 5 hr. of milking.

On arrival at the laboratory the samples were placed in a water-bath at 22° C., and the apparent lactic acid was determined by the ferric chloride method. Subsequently, determinations were made at 2·30 p.m. and then at 3-hourly intervals until the C.O.B. end-point was reached. To obtain apparent lactic-acid values on milk during the night, four 25 ml. aliquots of the sample were measured into sterile boiling tubes. These samples were incubated at 22° C. in a specially constructed water-bath (9) which automatically boiled each of the replicates in turn at 3 hr. intervals.

Samples of milk were examined to find if the heating stopped the bacterial action or affected the results. Samples which had been boiled sometimes gave slightly higher values than normal, but the increase was not great enough to prevent the use of this procedure. In Table 3 a sample of the results obtained from thirty winter and sixty-five summer samples is shown. All the results from samples which have been boiled are marked with an asterisk. It will be noted that these results are usually slightly higher than those from unheated samples but the differences are not important. Bacterial action was effectively stopped by the heating, so that lactic acid development ceased.

The results on each milk sample have been examined separately. Apparent lactic acid concentrations have been plotted against hours to C.O.B. end-point for each sample. Examination of the curves (not reproduced here) showed that the development of acidity followed a definite pattern. The initial values in milks varied slightly, but this variation was insignificant in comparison with the values obtained on milks containing developed lactic acid. The concentration of lactic acid in all cases remained constant at its initial value for some hours before increasing. This can be seen from the data in Table 3. In 80% of the summer samples this increase occurred 7–8 hr. before the C.O.B. end-point. Once the development of acidity began, its production was rapid. From graphs the times to the C.O.B. end-point were obtained at apparent lactic-acid concentrations in the milk of 0·02, 0·03, 0·04, 0·05, 0·06, 0·075, 0·09, 0·105, 0·125, 0·145 and 0·170%. Figs. 5*a* and *b* show the number of samples at the various keeping qualities (measured to the nearest hour) for each of these values for the winter and summer samples, respectively. The average keeping quality and the limits within which 80% of the samples fall for each reading were calculated and the values plotted in Figs. 6*a* and *b*. So that the reliability of the curves can be assessed the numbers of samples on which the curves were based are shown in Tables 4*a* and *b*.

The choice of that value of apparent lactic acid which would differentiate between a milk which is acceptable and one which should be rejected is governed by two main considerations. First, no milk should be rejected as a result of a high initial lactic-acid content (see section on late-lactation milk). Second, the value chosen must reject samples several hours before the C.O.B. end-point, but must not reject milks which still have a good life.

Dealing with the first point, apparent lactic-acid determinations were made on late-lactation milk, and it was decided that no sample of milk with less than 0·03% should be

rejected. Although this value was exceeded by two samples of fresh late-lactation milk, it was felt that a safe margin had been left, as late-lactation milk is usually well diluted with normal milk on arrival at the creamery. Of the samples of herd milk examined both in winter and summer, no sample which did not contain developed lactic acid had a value greater than 0·027%. The next highest value was 0·023%.

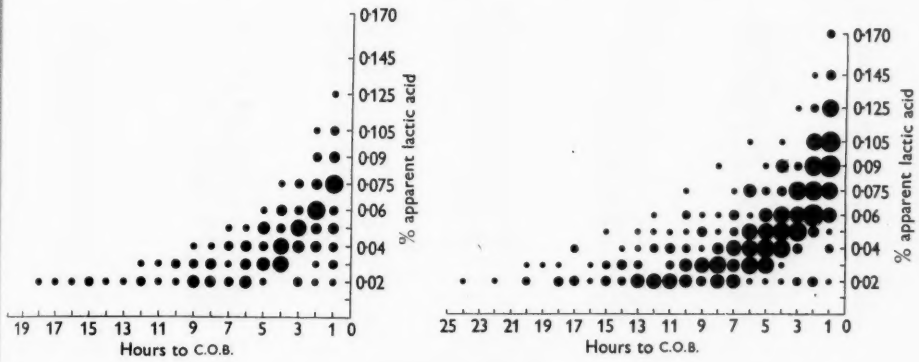

Fig. 5*a*. The relationship of apparent lactic acid in winter samples to the life of the milk. The smallest spot represents one sample by area.

Fig. 5*b*. The relationship of apparent lactic acid in summer samples to the life of the milk. The smallest spot represents one sample by area.

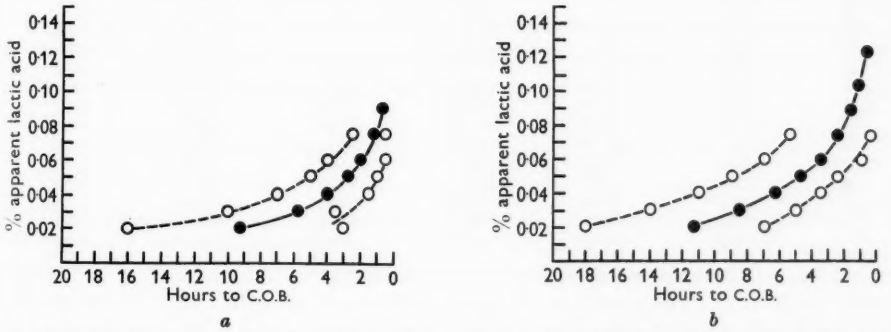

Fig. 6*a*. The relationship between average keeping quality and apparent lactic-acid concentration for winter samples (see Table 4). Full circles, average; open circles, 80% limits.

Fig. 6*b*. The relationship between average keeping quality and apparent lactic-acid concentration for summer samples. Full circles, average; open circles, 80% limits.

Considering 0·03% apparent lactic acid as a possible value at which to reject, it was seen from Figs. 6*a* and *b* that the average keeping qualities for winter and summer milks, from the time of reaching this value, are $5\frac{3}{4}$ and $8\frac{1}{2}$ hr., and that 80% of the samples had keeping qualities from $2\frac{1}{2}$ to 10 and 5 to 14 hr., for winter and summer samples, respectively. However, of the summer samples with more than 11 hr. keeping quality on reaching this value, it was found that 80% had a total keeping quality of more than 42 hr. These milks would still have a residual life of more than 24 hr. when arriving at the

Table 3. *Apparent lactic-acid concentrations measured at 3-hourly intervals until* C.O.B. *end-point*

(*a*) Winter samples

Hours to C.O.B.	% apparent lactic acid in samples no.					
	10	14	17	20	23	30
48	—	—	0·016	—	—	—
45	0·014	—	0·017*	0·019*	—	0·015
42	0·015	—	0·018*	0·019*	—	0·015
39	0·016	0·013	0·017*	0·019*	—	0·017*
36	0·018*	0·014	0·016*	0·023*	—	0·015*
33	0·019*	0·016*	0·016	0·018	—	0·015*
30	0·020*	0·016*	0·015	0·019	—	0·017*
27	0·020*	0·014*	0·018	0·019	0·015*	0·017
24	0·016	0·016*	0·017	0·020	0·015*	0·015
21	0·017	0·014	0·023*	0·020*	0·017*	0·016
18	0·020	0·015	0·025*	0·022*	0·019*	0·019
15	0·014	0·015	0·032*	0·022*	0·020	0·022*
12	0·026*	0·016	0·035*	0·023*	0·023	0·023*
9	0·035*	0·019*	0·024	0·025	0·030	0·023*
6	0·052*	0·024*	0·044	0·028	0·040	0·025*
3	0·079*	0·031*	0·058	0·030	0·064*	0·026
0	0·138	0·062*	0·088	0·037	0·094*	0·032

(*b*) Summer samples

Hours to C.O.B.	% apparent lactic acid in samples no.					
	26	32	33	38	42	46
48	—	—	0·016*	—	—	—
45	—	—	0·016*	—	—	—
42	0·017	—	0·019*	—	—	—
39	0·016	—	0·017*	—	—	—
36	0·020	—	0·015	—	—	—
33	0·017*	0·016	0·015	—	0·015	0·016
30	0·017*	0·015	0·015	—	0·015	0·016*
27	0·019*	0·015	0·014	0·019	0·015	0·016*
24	0·018*	0·010*	0·018*	0·023	0·016*	0·016*
21	0·018	0·017*	0·018*	0·020*	0·015*	0·017*
18	0·019	0·017*	0·020*	0·020*	0·016*	0·017
15	0·027	0·017*	0·019*	0·023*	0·019*	0·019
12	0·041	0·020	0·020	0·020*	0·016	0·023
9	0·054*	0·021	0·022	0·038	0·018	0·033
6	0·064*	0·030	0·032	0·041	0·023	0·046*
3	0·083*	0·040	0·049	0·081	0·044	0·061*
0	0·123*	0·050*	0·096*	0·206	0·088*	0·082*

* Determinations made on boiled samples from automatic C.O.B. bath.

creamery and would therefore be inspected before any acidity had begun to develop. Thus, on this basis milk rejected at 0·03% apparent lactic acid would have a keeping quality of less than 12 hr.

Of ninety-five samples examined 6% had reached the C.O.B. end-point before 0·03% lactic acid had developed, a further 3% before 0·04%, 5% before 0·05% and 4% before 0·06%. The higher the rejection level is placed the fewer the samples which would be detected before the C.O.B. end-point.

It is therefore suggested that 0·03% is the earliest suitable value at which to reject after considering all the above factors.

The six samples which would not have been detected, i.e. those which reached the C.O.B. end-point before they had developed 0·03% lactic acid, had keeping qualities of 26, 31, 32, 34, 37 and 42 hr. Four of these samples were taken in the summer and two in

Table 4. *Relationship between average keeping quality and apparent lactic acid (see Figs. 6a, b)*

(*a*) Winter samples

% Apparent lactic acid in milk	No. of samples	Average K.Q. (hr.)	80% Limits of K.Q. (hr.)
0·02	30	9½	3–16
0·03	28	5¾	3½–10
0·04	25	4	1½–7
0·05	23	2¾	1–5
0·06	19	2	½–4
0·075	17	1½	½–3
0·09	8	¾	0–2
0·105	3	1	—
0·125	3	0	—
0·145	1	0	—
0·170	—	—	—

(*b*) Summer samples

0·02	62	11⅓	7–18
0·03	60	8½	5–14
0·04	60	6½	3½–11
0·05	58	4¾	2½–9
0·06	57	3½	1–7
0·075	50	2½	½–5½
0·09	43	1¾	—
0·105	29	1½	—
0·125	18	¾	—
0·145	6	¾	—
0·170	4	½	—

the winter. The reasons for the early clotting of these samples at low acidity need further investigation. The frequency distribution of keeping quality values for both the winter and summer samples tested was examined and found to be normal; there was no preponderance of either high- or low-keeping quality milks.

Relationship between acidity development and bacteria present in milk samples

Samples taken from cans on the creamery platform and brought directly to the laboratory had apparent lactic-acid concentrations which did not alter appreciably until 9–12 hr. before the C.O.B. end-point was reached. It was not known whether this long delay before lactic acid could be detected was due to: (*a*) the lag phase of the bacteria, (*b*) quantities of lactic acid being too small for detection, or (*c*) no lactic acid being formed while the bacteria were actively reproducing, but only when a maximum population had been developed.

Accordingly, samples from cans at the creamery were incubated at 22° C. in the laboratory and the types of organisms present determined at 3-hourly intervals by plating the milks on double indicator agar (10). Countable plates were taken as those with 10–100 colonies, as the differentiation between acid or alkali-producing colonies was interfered with when larger numbers were present.

Apparent lactic-acid determinations were made at each time of plating and the results are shown in Fig. 7, where $\log_{10}$ count of acid producers and the lactic-acid concentration are plotted against time to C.O.B. end-point.

The lag phase of the bacteria lasts only a few hours. Acid-forming bacteria were increasing about 30 hr. before the C.O.B. end-point. Maximum numbers had by no means

been reached when the lactic-acid development was detected and from an examination of the data it appeared that the metabolism of the acid-forming bacteria had not changed and that lactic acid was in fact formed, but in quantities which could not be detected by the foregoing method.

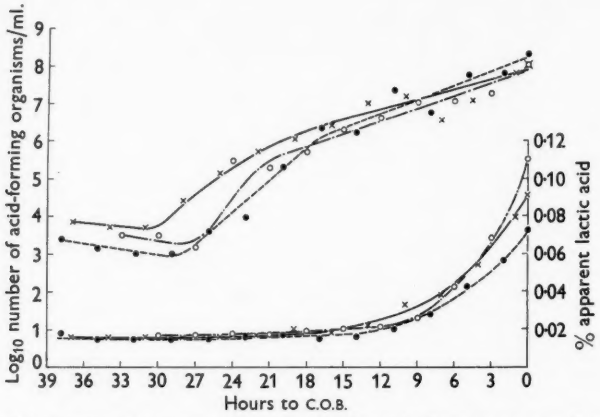
Log10 number of acid-forming organisms/ml.
0 1 2 3 4 5 6 7 8 9
0·02 0·04 0·06 0·08 0·10 0·12
% apparent lactic acid
39 36 33 30 27 24 21 18 15 12 9 6 3 0
Hours to C.O.B.

Fig. 7. The relationship between the number of acid-forming bacteria, hours to C.O.B. and apparent lactic-acid content on three selected samples. ×——×, sample 1; O—·—O, sample 2; ●– – –●, sample 3.

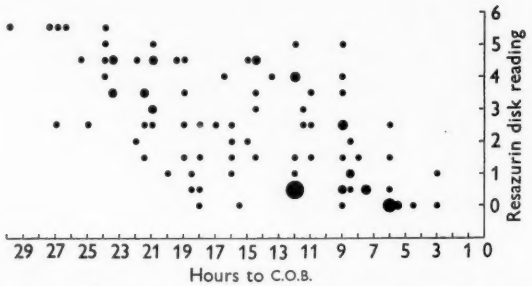

Fig. 8. The relationship between time to C.O.B. at 22° C. and resazurin disk reading after 10 min. at 37° C. The smallest dot represents one sample by area.

Relationship of resazurin disk readings with keeping quality

There are varied reports in the literature of the relationship of the 10 min. resazurin test to keeping quality. Davis (11) and Thomas *et al.* (12) gave results much lower than the samples we had examined, and it was decided to carry out 10 min. resazurin determinations on a number of the samples which were also examined for lactic acid at three-hourly intervals. Unfortunately, these determinations were only made during the period 9 a.m. to 6 p.m. and in consequence no samples have a complete comparison of disk readings to hours to C.O.B., but the results obtained which are given in Fig. 8 and Table 5 indicate that the residual life of a milk after it has reached a disk reading of $3\frac{1}{2}$ and 0 is longer than reported by Thomas (7·9 and 1·5 hr., respectively, at 22° C.) The results suggest that, on average, a sample takes 9 hr. to fall from a disk reading of $3\frac{1}{2}$ to 0. On individual samples the time is probably much shorter, 5–6 hr., although our results on this are sparse.

Thomas's results were obtained on a large number of samples taken from creameries during a hot summer, whereas our samples were taken during a relatively cool summer, this may in part account for the differences in our findings.

Table 5. *The relationship between resazurin disk reading and keeping quality*

Resazurin disk reading	No. of samples	Hours to C.O.B.		
		Mean	Lowest	Highest
5½–5	9	22	9	30
4½–4	19	18	9	25
3½–3	12	17	9	23
2½–2	18	15	6	27
1½–1	17	13	3	21
½–0	25	8	0	18

The effect of abnormal milk on the test

It is known that fresh raw milk contains small quantities of lactic acid, but it is not known whether this value is affected by abnormal conditions of the cow. Samples from individual cows were examined (*a*) soon after calving, (*b*) at regular intervals during lactation (to see if there is any change due to the period of lactation or yield), and (*c*) with mastitis.

(*a*) *Colostrum*

Samples of colostrum milk were taken from six cows for the first 6 days after calving. For the first 3–4 days the colostrum clotted on boiling, but the milk of the 5th and 6th days appeared normal and had initial lactic-acid values slightly lower than average.

(*b*) *Period of lactation*

Samples of milk were taken from eight cows at approximately four-weekly intervals from March until the cows were dried off between September and November. The samples were examined directly after milking, and the results expressed as apparent lactic acid. These values and the relationship between the yields and apparent lactic-acid concentrations are shown in Fig. 9 and Table 6.

As there appeared to be larger values of lactic acid in the late-lactation milk (previously noted by Ling (3)) particularly when lower yields were obtained, further samples of milk were obtained from cows near the end of lactation, and the lactic acid was determined by two methods, i.e. with ferric chloride and with *p*-hydroxy diphenyl. The values obtained are given in Table 7.

There appears to be an inverse relationship between the yield and the apparent lactic-acid content, but high values of lactic acid are not obtained from all cows giving low yield.

On examining the results of summer milks taken from the creamery it was found that five samples had higher than average initial apparent lactic-acid concentrations which remained nearly constant until developed lactic acid was measurable as in normal milks.

One sample was a milk from a herd of fifty cows. All of these cows were being dried off at the same time and the average yield per cow was down to ½ gal. per day. A second sample from this herd was taken in November when the average yield per cow was 3 gal. and the initial lactic acid was then found to be normal.

Another sample, no. 40, was taken from a herd where ten out of twenty-six cows had yields of less than 1 gal. per day; some of them giving only a total of 3–3½ lb.

The third sample was from a small herd of six cows, one of which was nearly dry.

Table 6. *Relationship between period of lactation, yield and apparent lactic-acid concentration*

Cow	Apparent lactic-acid and yield on 26. iii. 56	24. iv. 56	5. vi. 56	4. vii. 56	31. vii. 56	5. ix. 56	26.ix.56.	3. x. 56	
1	0·012 (6½)	0·012 (5)	0·013 (6¾)	0·015 (6½)	0·020 (4½)	0·021 (3)	0·024 (2½)	*1. x. 56*	—
2	0·012 (7½)	0·012 (7¾)	0·012 (7)	0·015 (8½)	0·016 (7½)	0·018 (4½)	—	0·016 (6)	*1. xi.* 56
3	0·011 (7½)	0·012 (7½)	0·011 (8¾)	0·014 (8½)	0·015 (7)	0·014 (5½)	*20.ix.56*	—	—
4	0·012 (8¾)	0·013 (8¾)	0·012 (9¼)	0·014 (10¼)	0·017 (5¾)	0·017 (3¾)	*13.ix.56*	—	—
5	0·013 (5¾)	0·014 (6½)	0·013 (6½)	0·017 (4½)	0·020 (5)	0·019 (4½)	—	0·018 (6)	*8.xi.56*
6	0·014 (7)	0·014 (7¾)	0·012 (8¾)	0·017 (7¾)	0·020 (6¾)	0·019 (6¾)	—	0·017 (8½)	*1.xi.56*
7	0·012 (7)	0·015 (2½)	0·012 (5½)	0·015 (5)	0·015 (4¾)	0·020 (2½)	*6.ix.56*	—	—
8	0·013 (7½)	0·014 (5½)	0·014 (6½)	0·017 (7)	0·020 (3¾)	*30.viii.56*	—	—	—

The values in parentheses are yields in lb.
The date on which each cow was dried off is given in italics.

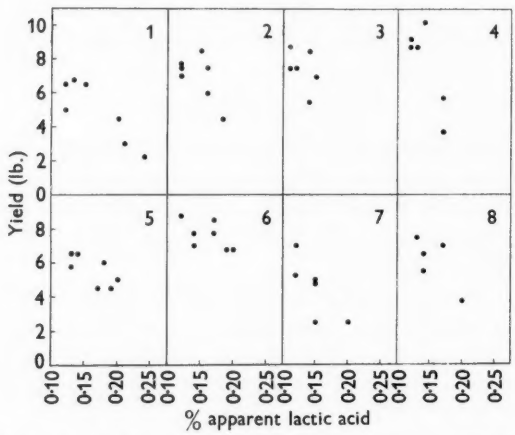

Fig. 9. The relationship between yield and apparent lactic-acid concentration with eight individual cows.

The fact that higher initial values of apparent lactic acid have been found on individual samples of late-lactation milk, and also on herd samples, where it was subsequently found that a high proportion of the cows were being dried off, means that care must be taken in fixing the apparent lactic-acid concentration, above which milks would be rejected, so that no sample of fresh raw milk should be rejected because it has a high initial apparent lactic-acid content.

Table 7. *Values of apparent and true lactic acid in late-lactation milk*

Cow	No. of days before drying off	Meal	Yield (lb.)	Apparent lactic acid (by ferric chloride) (%)	True lactic acid (by *p*-hydroxy diphenyl) (%)
a	9	E	1	0·015	0·0015
b	8	E	1¼	0·043	0·0152
c	8	E	1½	0·037	0·0133
d	4	E	1½	0·027	0·009
a	8	M	1½	0·015	0·0017
e	4	E	2	0·025	0·006
c	3	M	2	0·023	—
d	3	M	3	0·022	0·0075
f	11	E	3½	0·016	0·0029
g	29	E	4	0·014	0·0013
h	23	E	5	0·022	0·0043
i	20	E	5	0·014	0·002
f	10	M	5¼	0·015	0·0019
g	36	E	6	0·014	0·0022
h	23	E	6	0·021	0·0049
i	19	M	6½	0·014	0·0017
g	28	M	7½	0·015	0·0017

(*c*) *Mastitis*

Milk from cows suffering from mastitis may escape rejection providing the milk is normal in smell and appearance. Accordingly, several determinations of apparent lactic acid were made on milks from cows with mastitis, to see whether abnormal results were obtained.

The samples were taken from two sources. (1) A herd of heifers which is kept for experimental purposes at the N.I.R.D. (2) A herd of cows which recently had an outbreak of mastitis. In both cases one morning and one evening sample was taken from each cow. The severity of the mastitis infection has been classified as mild, medium, or severe.

It is evident from the results in Tables 8 and 9 that none of the samples gave an excessively high apparent lactic-acid value, and none would have been rejected.

Table 8. *Samples of milk from cows suffering from mastitis in an experimental herd*

Cow	Type of case	Time of sampling	% apparent lactic acid	Yield (lb.)
1	Medium	25. i. 57 Evening	0·016	8
		26. i. 57 Morning	0·014	14
2	Severe	25. i. 57 Evening	0·018	15¼
		26. i. 57 Morning	0·015	27
3	Severe	26. i. 57 Morning	0·016	20½
		26. i. 57 Evening	0·016	13
4	Medium	26. i. 57 Evening	0·016	9½
		27. i. 57 Morning	0·015	15
5	Medium	13. ii. 57 Evening	0·014	8
		14. ii. 57 Morning	0·014	12
6	Medium	27. ii. 57 Evening	0·022	3
		28. ii. 57 Morning	0·020	4
7	Slight	27. ii. 57 Evening	0·020	5
		28. ii. 57 Morning	0·015	9
8	Medium	27. ii. 57 Evening	0·018	6
		28. ii. 57 Morning	0·016	10

Colour measurement

In the experimental work in the laboratory on the relationship between apparent lactic acid and keeping quality, the intensity of the yellow colour produced when 1% ferric chloride was added to milk filtrate was determined on the Spekker absorptiometer, a method unsuitable for use in a creamery.

As one of the objects of this work has been simplicity of testing, the matching of colour with a disk in a Lovibond comparator was tried first.

Table 9. *Samples of milk from cows suffering from mastitis in a normal herd*

Cow	Type of case	Time of sampling	% Apparent lactic acid	Yield (lb.)
1	Slight	15. ii. 57 Evening	0·014	8¼
2	Severe	12. ii. 57 Evening	0·021	4
		16. ii. 57 Morning	0·018	8¼
3	Slight	15. ii. 57 Evening	0·013	1¾
		16. ii. 57 Morning	0·014	3
4	Medium	15. ii. 57 Evening	0·011	8½
		16. ii. 57 Morning	0·011	12
5	Severe	15. ii. 57 Evening	0·011	9¾
		16. ii. 57 Morning	0·011	15¼
6	Medium	15. ii. 57 Evening	0·011	13¾
		16. ii. 57 Morning	0·009	18
7	Severe	15. ii. 57 Evening	0·014	8½

From an examination of the average curve for summer samples, Fig. 6*b*, it was decided that six colour intensities produced by the addition of 2 ml. of 1% ferric chloride to 10 ml. of fresh raw milk filtrate containing varying quantities of added lithium lactate should be used to prepare disks.

The milk chosen for the preparation of these solutions was from a herd known to give milk with normal initial apparent lactic-acid values. Six solutions were prepared from this milk which produced the required colour intensities with 2 ml. of 1% ferric chloride solution.

The yellow colours produced by these solutions were distinguishable from one another when placed in test-tubes in a rack, but it was felt that it might be desirable, to simplify matching, to try to produce a change in colour rather than a change in intensity of colour.

Ling's solutions A, B and C which contain salicylic acid, potassium ferrocyanide and ferric chloride, and which produce a colour change from violet-blue through colourless to grey, green and yellow-green over various lactic-acid concentrations, were modified in an attempt to get a colour change over the range of acid concentrations required, but it was not possible to obtain a wide enough range.

More success was obtained when phenol and ferric chloride were added (13), and a colour change from violet through grey to yellow which then increased in intensity, was obtained by the addition of 2 ml. 1% ferric chloride and 1 ml. of 6% phenol to the filtrate. Using these quantities of reagents a grey colour was produced in the solution which contains the concentration of apparent lactic acid which we propose as the earliest rejection level.

Two disks were prepared. Disk A had yellow colours matching those obtained by adding 2 ml. of 1% ferric chloride solution to 10 ml. of the six solutions (see Table 10). Disk B had a colour change from violet to yellow matching colours obtained by adding

Table 10. *Values used in the preparation of a colour matching disk*

Disk			
A (no.)	B (no. and colour)	Spekker reading	Apparent lactic acid (%)
1	1 Violet	0·80	0·02
2	2 Grey	0·75	0·03
3	3 Yellowish grey	0·70	0·04
4	4 Greyish yellow	0·60	0·06
5	5 Yellow	0·50	0·09
6	6 Intense yellow	0·42	0·12

2 ml. of 1% ferric chloride and 1 ml. of a 6% phenol solution to 10 ml. of the chosen solution.

To test the ease of matching the two sets of colours, twenty samples of milk taken from cans at creameries were incubated at 22° C. and examined at three-hourly intervals until the C.O.B. end-point. 10 ml. aliquots of the milk filtrates obtained were examined by three methods.

(1) 2 ml. of 1% ferric chloride were added and readings were made on the Spekker using a 1 cm. cell and 601 violet filters.

(2) 2 ml. of 1% ferric chloride were added and the colour was matched visually against disk A in a Lovibond comparator. There was no difficulty in matching the solutions, and the results were expressed as a whole number if an exact match was obtained, + or − if the colour intensity of the solution was slightly greater or less than that of a disk number, and ½ if between a 'plus' and 'minus'.

(3) 2 ml. of 1% ferric chloride and 1 ml. of a 6% phenol solution were added and the resulting colour was matched against disk B. Again, readings were made to the nearest whole number, plus, minus or ½.

All colour matchings were made by two observers, and the results are expressed graphically in Figs. 10*a*, *b*. Disk readings have been plotted against Spekker readings. Where the readings obtained by the two workers agreed the value is shown by a single dot. When different disk values were obtained, the two values are represented by dots joined by a line. The dotted lines represent the positions of disks 1–6.

The results in Fig. 10*a* indicated that the intensity of yellow colour in no. 2 of disk A was slightly low, and as this value is critical from the point of view of rejection the colour was checked by three observers against five solutions giving Spekker readings from 0·779 to 0·727. The results are given in Table 11, which confirm that the colour of disk A, no. 2, in the first trial disk tested was slightly lower than it should have been. Subsequently, the disk has been adjusted.

Table 11. *A check of disk A, no. 2, by three observers*

Solution	Spekker reading	Disk reading with observers		
		A	B	C
1	0·779	2	2⁻	2
2	0·767	2+	2⁻ almost 2	2
3	0·750	2+	2+	2+
4	0·745	2½	2+	2+
5	0·727	3⁻	2½	2½

Far less difficulty was met in matching the yellow colour with disk A than the violet to yellow change with disk B. The figures show that there is less scatter of results with disk A.

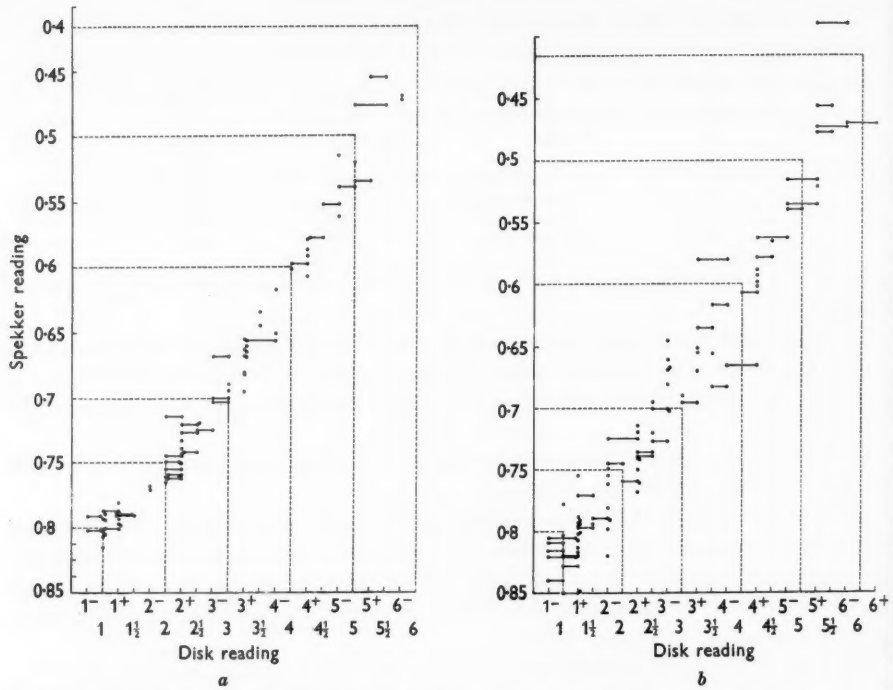

Fig. 10*a*. Observations on the relationship of disk readings to Spekker readings by two observers using disk A. Dots joined by a line represent different values by two workers on a single sample.

Fig. 10*b*. Observations on the relationship of disk readings to Spekker readings by two observers using disk B. Dots joined by a line represent different values by two workers on a single sample.

DISCUSSION AND CONCLUSIONS

The rapid test described for apparent lactic acid in milk is simple and could be done in most creamery laboratories with a little practice. It should be possible to complete a single test in about 5 min. and probably six tests could be done simultaneously in about 10 min. The colour of the ferric lactate complex is stable for at least 20 min. at laboratory temperature, thus allowing several tests to be done simultaneously.

Although the test is simple, care is necessary in carrying it through. Little latitude can be permitted in the accuracy with which the reagents are prepared or the amounts measured in the test. Distilled water from a still with a glass or block tin condenser must be used.

It is possible to distinguish by eye differences of 0·02, 0·03 and 0·04% lactic acid, but when cans of milk are being considered for rejection such judgement is not satisfactory. Comparison can be made much more accurately by the use of a suitable comparator and standard disk, and special test tubes made of colourless glass which are essential. If the test for apparent lactic acid were to be used for the rejection of milk, strict precautions should be observed by all users of the test.

The 'accuracy' of the test has been assessed on its relationship to the C.O.B. end-point. This relationship is good but not perfect, because there are factors other than acidity

which cause a clot on boiling. Therefore, a test for developed acidity cannot select all poor-keeping quality milks if the end-point of keeping quality is judged as C.O.B.

In this work 6% of the samples examined failed to develop as much as 0·03% lactic acid (the suggested earliest rejection level) before the C.O.B. end-point was reached. One-third of these samples had keeping qualities greater than 36 hr. at 22° C., and therefore would presumably cause little trouble on the creamery platform or elsewhere. However, we are left with 4% of the samples with a poor keeping quality which would fail to be detected by a test for apparent lactic acid.

We know little about such samples except that at the C.O.B. end-point they contain a large number of slow acid-producing organisms. All of these milks were tested by the 10 min. resazurin test which gave a normal advanced prediction of the C.O.B. end-point.

The extent of this work has been limited, and before much can be said the extent to which these anomalous samples occur in practice should be assessed by trials of this method at the creamery. Further, the mechanism of clotting at low acidity is one which should be fully investigated.

SUMMARY

The determination of apparent lactic acid has been used as a basis for a rejection test for raw milk. The method consists of precipitation of milk proteins with barium chloride, sodium hydroxide and zinc sulphate, and the addition of ferric chloride to the filtrate to produce the yellow colour of ferric lactate. Lactic acid is not solely responsible for the production of the yellow colour, which, nevertheless, gives a good relationship with keeping quality of milk (measured as hours to the C.O.B. end-point at 22° C.) and the values have been expressed as 'apparent lactic acid'.

The relationship of winter and summer milks to keeping quality has been studied, and a value of 0·03% apparent lactic acid in milk is equivalent to an average keeping quality of $5\frac{3}{4}$ and $8\frac{1}{2}$ hr. for winter and summer milks, respectively. A value of 0·03% is recommended as the earliest value of apparent lactic acid at which milk could be rejected.

The apparent lactic acid in colostrum and late-lactation milk and in milk from cows suffering from mastitis has been determined, and only in late-lactation milk were the values found to be significantly higher than usual in fresh raw milk, and an inverse relationship between yield and apparent lactic acid is suggested.

Permanent glass matching disks have been prepared for use in a Lovibond comparator. This permits the intensity of the yellow colour produced with 1% ferric chloride to be determined and the apparent lactic acid in milk estimated.

Grateful acknowledgement is made to the management and staff of the Dairy Department of the Reading Co-operative Society and the Farmer's Clean Milk Dairy, Reading, and local milk producers for supplying samples for experiments; to the N.M.T.S. staff in Reading for help in finding suitable farmers, and to the Dairy Husbandry Department of the N.I.R.D. for information about and samples of abnormal and late-lactation milk. Our particular thanks are due Miss Marie Gruber for technical assistance, to Dr N. J. Berridge for the suggestion and help on the work on pH change as an indication of keeping quality (given in the appendix), and to Dr A. T. R. Mattick for the advice given in this work.

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APPENDIX

INVESTIGATION OF THE POSSIBILITIES OF pH CHANGE AS AN INDICATION OF KEEPING QUALITY OF MILK

Samples of milk which were taken from cans on arrival at creameries and were brought to the laboratory about 6 hr. after milking were divided into two parts. To one-half (*a*) merthiolate was added to stop bacterial action, and the other half (*b*) was untreated. All samples were then incubated at 22° C. When the milk had reached 22° C. readings were made on the Vibron electrometer using a normal glass electrode, and 'apparent lactic acid' was determined by the ferric chloride method. Readings were subsequently made at intervals until C.O.B. end-point was reached.

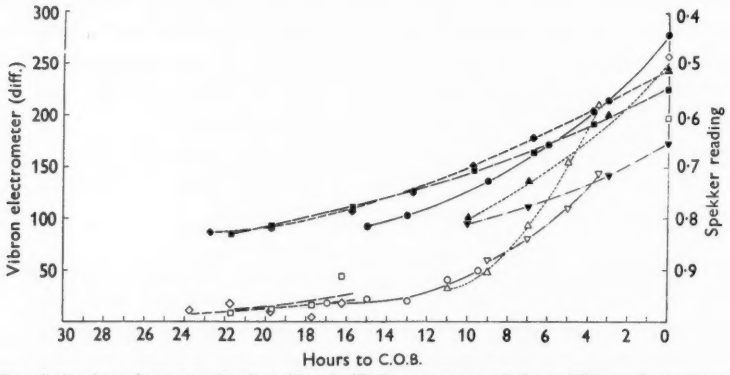

Fig. 11. The relationship of apparent lactic acid and pH change, as recorded on a Vibron electrometer, to C.O.B. Circles, sample 1; diamonds, sample 2; inverted triangles, sample 3; upright triangles, sample 4; squares, sample 5. Black dots, apparent lactic acid; white dots, pH change as recorded on Vibron electrometer.

Fig. 11 shows the apparent lactic acid plotted against hours to C.O.B., and also the difference in readings on the Vibron electrometer between subsamples (*a*) and (*b*) of each milk sample plotted against hours to C.O.B. It is seen that the curves of the two sets of

determinations are similar for each sample. This was expected as both are dependent on acid production.

As the initial pH of fresh raw milk will probably be between 6·5 and 6·7, acid development can only be detected satisfactorily as a difference between two consecutive pH determinations. It had been hoped that these differences in a short time interval (5 to 10 min.) would have been large enough to have been detected on the Vibron electrometer, when acidity was developing, and that it could have been used as a means of showing acid development. From the graphs it is seen that the change in 10 min. is only about 1 unit ($\approx 0{\cdot}002$ pH). This value is considerably less than the limits of error of each reading.

It has, therefore, been decided that this instrument in its present form cannot be used to detect acid development in milk in a short space of time.

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694. OBSERVATIONS ON THE CELL COUNT AND SOLIDS-NOT-FAT CONTENT OF COWS' MILK

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(With 2 Figures)

In recent years there has been a progressive decline in the solids-not-fat (S.N.F.) content of the milk supply in Britain (Provan (1)). While feeding, breeding and management are clearly important factors to be considered in elucidating causes of a low S.N.F. in milk, it would appear that the influence of subclinical mastitis in this connexion has not been fully recognized. Kay (2) in a review of the S.N.F. problem has stated that a careful survey showed that quantitatively mastitis was a minor factor in diminishing the S.N.F. content of the bulk milk of the national supply, but it might, in single seriously infected herds, be a major cause of low quality. Recently Griffiths & Featherstone (3) in an investigation into the nature of the S.N.F. problems in the west Midlands of England reported no abnormal incidence of *Streptococcus agalactiae* infections in the herds under investigation, and concluded that there was no undue incidence of disease of the udder. On the other hand, Malcolm, King & Campbell (4,5) maintained that the cell count of milk is the most reliable criterion of mammary inflammation, and McFarlane, Blackburn, Malcolm & Wilson (6) in a comparison of ante-mortem and post-mortem findings in bovine mastitis, showed that the ante-mortem cell-count test agreed with the post-mortem histological examination of the udder in 92% of cases, whereas culture tests of the milk agreed in only 56%. On the basis of the cell-count test Laing & Malcolm (7) found that a non-specific *subclinical* form of mastitis constituted over 38% of the mastitis in affected cows and over 54% of the mastitis in affected quarters, and suggested that this *largely unrecognized* form of the disease can have an important influence on the composition of the milk. Accordingly, it seemed desirable to determine the relationsh whichip may be found between the cell count and the S.N.F. content of milk.

Commencing in December 1954 an investigation on the chemical composition of the milk from cows in the Ayrshire herd on the College farm at Craibstone has been undertaken by one of us (W.M.), and the opportunity has been taken to make concurrently cell counts on the milk from the same cows. The results of this comparison form the principal part of the present report.

In this investigation two types of sample were examined:

(1) quarter samples of the first few streams of milk from each teat which are referred to as quarter samples of fore-milk,

(2) samples taken from the milk bucket on completion of milking which are designated individual bulk samples.

The survey may be divided into three periods. In the first period (between December 1954 and July 1955) the S.N.F. content of monthly samples of milk from each cow in the herd was compared with the cell count obtained from individual quarter samples of fore-

milk taken on or about the same day. In the second period (between August 1955 and December 1956) the S.N.F. content of monthly individual bulk samples from each cow in the herd was compared with the cell count obtained from the same individual bulk milk sample. In the final period (between March and May 1957) a similar comparison was made between the S.N.F. content and cell count of individual bulk samples taken from cows in eleven herds in Aberdeenshire and Kincardineshire. The breeds from which the samples were taken in this period included Ayrshire, Friesian and Friesian crosses, and a small number of Jerseys.

Laing & Malcolm (7) have suggested that a total cell count >250,000/ml. in a *quarter* sample of fore-milk from a cow in mid-lactation should be regarded as suspicious. Since, in the second and third periods, we were estimating the cell counts of *bulk* individual cow samples, it was necessary to determine the number of cells which could be regarded as abnormal in such samples. Accordingly, during the course of the investigation a comparison was made of the cell counts obtained from (*a*) quarter samples of fore-milk, and (*b*) individual bulk samples taken from the milk bucket at the conclusion of milking.

While this work was concerned primarily with a comparison of the cell count and S.N.F. of milk, the fore-milk quarter samples taken in periods 1 and 2 were also examined bacteriologically for the presence of typical mastitis bacteria.

METHODS

The monthly samples from each cow in the College herd in the first period (December 1954 to July 1955) consisted of morning and evening samples taken from the milk bucket at successive milkings. A bulked sample of morning and evening milk was used for determination of the total solids content gravimetrically and the fat content by the Gerber method. The S.N.F. content was obtained by difference. The quarter samples of fore-milk for cell-count determination were taken at the same milking, or within a day or so of the bulk sample being taken for chemical analysis. (It was considered that there would be little difference in either the cell count or S.N.F. % of the milk in this short interval.) In the second period at Craibstone (between August 1955 and July 1956) the cell count was made on a sample taken from the bulked sample of morning and evening milk that was used for the S.N.F. determination. In the third period (betweeen March and May 1957) only evening bulk samples were taken and the cell count and S.N.F. determination were made on the same sample.

The cell counts were estimated by Breed's microscopic method, but a standard platinum loop was used to withdraw 0·01 ml. of milk as described by McKenzie & Lambert (8) and Blackburn, Laing & Malcolm (9). Newman's reagent was used to stain the slides. The bacteriological examination of the quarter samples consisted in stroking a loopful of fore-milk on plain blood agar and incubating for 24–48 hr. at 37° C. The further examination of these plates was made by the Veterinary Investigation Department of the College.

RESULTS

In order to eliminate those animals where a high cell count might have been due to colostrum or late lactation milk, the comparison of cell count and S.N.F. has been confined to samples taken between the second and ninth months of lactation. As mentioned before,

Laing & Malcolm (7) have suggested that a cell count >250,000/ml. in a quarter sample of fore-milk from a cow in full milk should be regarded as suspicious. In considering the results of samples taken during the first period, therefore, a cell count of this order in one or more quarters has been regarded as abnormal, and the samples have been placed in two groups according to whether the cell count was below or above this figure. The S.N.F. percentages have been grouped in four ranges, namely, >9·5, 9·5–9·0, 8·9–8·5 and <8·5. On this basis the comparison is given in Table 1 of the percentage in each S.N.F. group of 321 samples, with normal or high cell counts taken in the first period. It will be noted that whereas 6·7% of samples with a normal cell count had a S.N.F. % <8·5, the corresponding figure was 22·7% for samples with a high cell count.

Table 1. *Comparison of solids-not-fat group and cell content (quarter samples) from a single herd (December 1954 to July 1955)*

Cell content	% in each S.N.F. group				
	<8·5	8·5–8·9	9·0–9·5	>9·5	Total
Normal	6·7	18·2	40·0	35·1	225
High	22·7	42·5	25·6	9·2	96
				Total	321

Normal cell content = <250,000/ml. in all quarters.
High cell content = >250,000/ml. in one or more quarters.

In the second period of the investigation the cell count and S.N.F. determination were made on the same sample of individual bulk milk. In a cell count of individual bulk milk, two factors will influence the number of cells in 0·01 ml., namely, the severity of the infection (or the number of quarters affected) and the amount of dilution with normal milk of a low cell count from the healthy quarters. It was assumed that a cell count >100,000/ml. for a bulk sample from a cow in mid-lactation should be regarded as significant. To verify this assumption cell counts on quarter samples of fore-milk were made on all cows at Craibstone whose bulk cell count was >100,000/ml. Further, in a series of 100 random cow samples, cell counts were compared of (*a*) quarter samples of fore-milk, and (*b*) bulk samples taken from the milk at the end of milking. The results of these comparisons are set out in Table 2. From the Craibstone results it will be seen that of 163 bulk samples with cell counts >100,000/ml. sixteen (9·8%) showed no quarter with a high cell count. In the 100 random cow samples, eight cows with cell counts >100,000/ml. in bulk milk showed no quarter with a high cell count, but five, where the bulk cell count was <100,000/ml., showed a high cell count in one quarter only. From these results it was considered that a cell count >100,000/ml. in bulk milk was abnormal, and this standard was used in the comparison of the S.N.F. and cell count tests in the second and third periods. It is worthy of note that McFarlane *et al.* (6) made use of such a standard in the examination of individual quarter milk.

In Table 3 is set out the percentage in each S.N.F. group of 774 samples with normal or high cell counts taken in the second period. It will be seen that in this period, whereas 10·5% of samples with a normal cell count had a S.N.F. content below 8·5%, the corresponding figure was 30·5% for the samples with a high cell count.

These results were compiled from monthly samples taken from a single herd of Ayrshires. It was thought desirable to ascertain whether a similar pattern would be found with samples drawn from a number of different herds and different breeds. Accordingly,

between March and May 1957, samples of bulk evening milk were taken from 375 cows in eleven herds. The results of this series of tests are recorded in Table 4 in which, as before, the percentage in each S.N.F. group is given of the samples with normal or high cell counts. In this series 21·6% of samples with a normal cell count had a S.N.F. <8·5, compared with 28·8% of samples with a high cell count.

Table 2. *Comparison of the cell counts of bulk milk and quarter samples of fore-milk from the same cows*

(A) 163 cow samples in College herd all with counts >100,000/ml. in bulk milk.

No. of quarters with cell counts >250,000/ml.	Cell count of bulk milk/ml. 100,000–150,000	150,001–250,000	>250,000	Total
None	8	3	5	16
1	11	2	6	19
2	17	13	18	48
3	13	14	19	46
4	4	9	21	34
			Total	163

(B) 100 random samples with varying bulk cell counts.

No. of quarters with cell counts >250,000/ml.	Cell count of bulk milk/ml. 1,000–50,000	50,001–100,000	100,001–150,000	150,001–250,000	>250,000	Total
None	51	13	4	2	2	72
1	2	3	2	1	5	13
2	0	0	1	0	5	6
3	0	0	0	2	3	5
4	0	0	0	1	3	4
					Total	100

Table 3. *Comparison of solids-not-fat group and cell content (bulk samples) from a single herd (August 1955–December 1956)*

Cell content	% in each S.N.F. group <8·5	8·5–8·9	9·0–9·5	>9·5	Total
Normal	10·5	37·7	37·2	14·5	607
High	30·5	34·1	23·8	11·4	167
				Total	774

Normal cell content = <100,000/ml. in bulk milk.
High cell content = >100,000/ml. in bulk milk.

The results recorded in Table 4, while still showing a higher proportion of S.N.F. <8·5% among the cows with high cell counts, did not show the big differences in this respect recorded in Tables 1 and 3. Examination of the individual herd results showed that the principal cause of this difference lay with the Friesian herds. The corresponding percentages for five Friesian and five Ayrshire herds are given in Table 5. It will be seen that whereas little difference was found in the percentage of low S.N.F. (<8·5%) in the samples with normal or high cell counts in the Friesian herds, a marked difference was found in the Ayrshire herds. Further examination of the Friesian samples with a S.N.F. <8·5% still showed little difference in the proportion between the normal and high cell count groups, either in the S.N.F. range 8·5–8·3 or <8·3%.

The bacteriological examination of the quarter samples from the College herd have been tabulated in Table 6. The main points which may be noted in this table were the high

incidence of staphylococcal infections and the large proportion of samples from which no organism of pathological significance was isolated. It should be pointed out, however, in connexion with these results that penicillin-therapy was being used in the herd for all known cases of mastitis, and this probably accounted for a number of the samples from which negative bacteriological findings were reported.

During the investigation a note was made of the number of lactations of each animal being tested. The average S.N.F. content for the cows while in each lactation has been calculated for all the samples with normal and high cell counts separately. These results

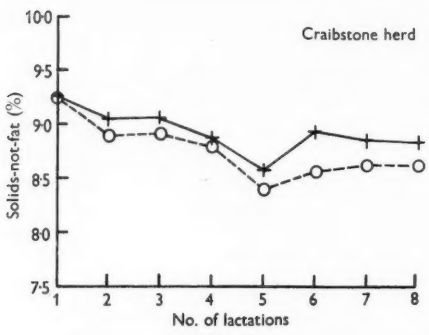

Fig. 1

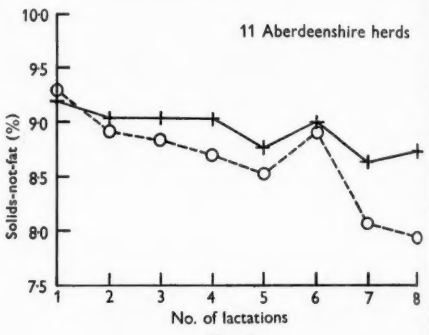

Fig. 2

Fig. 1. The influence of cell count on the S.N.F. calculated as a lactation average. The average lactation shown is based on monthly samples taken in the 2nd to 9th month of the lactation inclusive. ——+——, <100,000/ml.; ---○---, >100,000/ml.

	No. of samples								Total samples	
+	233	185	105	61	50	45	37	17	733	974
0	28	46	31	26	32	28	41	9	241	

Fig. 2. The influence of cell count on the S.N.F., calculated as a lactation average (based solely on a single test from all cows in that lactation). ——+——, <100,000/ml.; ---○---, >100,000/ml.

	No. of samples								Total samples	
+	67	77	44	18	17	12	12	6	253	367
0	22	27	28	10	7	9	3	8	114	

have been presented graphically in Fig. 1 for the College herd in period 2 and in Fig. 2 for the eleven herds in period 3. The small number of samples from Jersey cows (seventeen) taken during the latter period have been excluded from the results recorded in Fig. 2, since the S.N.F. content of these samples was much higher than for either the Ayrshire or Friesian samples, and the number of samples involved was too small for separate calculation to yield any valid conclusion.

DISCUSSION

In Britain at the present time the presumptive legal standard for the S.N.F. of milk is 8·5%. In this work attention has been directed primarily to those samples where the S.N.F. was below this figure, and the results in Tables 1, 3 and 4 have been purposely condensed for simplicity of presentation. It will be noted in Tables 1 and 3 that there was a marked increase in the percentage of samples with a S.N.F. content <8·5% in the high cell count group compared with the normal cell count group. This increase was not so

pronounced in Table 4. Tables 1 and 3 represent monthly samples from a single herd of Ayrshires sampled over periods of 7 months and 17 months, respectively, whereas Table 4 gives the results from 375 cows in eleven herds sampled on one occasion only. It is possible that the results in Tables 1 and 3 reflect the normal pattern in a single herd where subclinical mastitis is present. In Table 4, on the other hand, the relationship between cell count and S.N.F. has become obscured by other factors affecting S.N.F., e.g. different breeds and management, and this would seem to be confirmed by the difference in the figures for the Ayrshire compared with the Friesian herds recorded in Table 5.

Table 4. *Comparison of solids-not-fat group and cell content (bulk samples) from 11 herds (March–May 1957)*

Cell content	% in each S.N.F. group				Total
	<8·5	8·5–8·9	9·0–9·5	>9·5	
Normal	21·6	35·0	28·5	14·8	264
High	28·8	26·1	27·0	18·0	111
				Total	375

Normal cell content = <100,000/ml. in bulk milk.
High cell content = >100,000/ml. in bulk milk.

Table 5. *Comparison of solids-not-fat group and cell content (bulk samples) from cows in five Friesian and five Ayrshire herds*

Cell content	% in each S.N.F. group				Total
	<8·5	8·5–8·9	9·0–9·5	>9·5	
		Friesian herds			
Normal	27·4	29·6	36·3	6·6	135
High	28·1	26·7	36·6	8·5	71
				Total	206
		Ayrshire herds			
Normal	18·1	39·6	34·2	8·1	149
High	38·3	27·6	23·4	10·6	47
				Total	196

Normal cell content = <100,000/ml. in bulk milk.
High cell content = >100,000/ml. in bulk milk.

Table 6. *Incidence of typical mastitis organisms in 661 quarter samples with high cell counts*

Type of infection	%
Staphylococcal	265 (40·1)
Streptococcal or mixed	86 (13·0)
No specific organism isolated	310 (46·8)
Total	661

More detailed examination of our results has shown that a very wide scatter in S.N.F. may be found at any particular cell count level. It cannot be suggested, therefore, that milk with a high cell count will always have a S.N.F. content bordering on the present legal minimum or below it. But a high cell count is an indication of an abnormal condition in the udder, and the S.N.F. of milk from such an udder will be lower than it potentially could be. This is borne out by the average lactation S.N.F. recorded in Figs. 1 and 2. These graphs show the now well recognized decline which occurs in the percentage S.N.F. in successive lactations. It must be emphasized that the average S.N.F. shown in these graphs is not a complete lactation average, but the average calculated on the results

available for the period of the experiment. For example, in Fig. 1 the S.N.F. results of the first and late months of the lactation have been omitted since the cell counts for samples taken in these months were not used in our comparisons, and in Fig. 2 the average S.N.F. for each lactation has been calculated from a single test for each of the cows in that lactation. The curves in both figures follow a similar trend, but that of the high cell count follows a lower S.N.F. level. These results would appear to confirm that a broad correlation exists between the cell count and S.N.F. content. A feature peculiar to both Figs. 1 and 2 is the rise in the average S.N.F. between the 5th and 6th lactations. The possible explanation is that the disposal of uneconomic animals becomes more frequent about this age and the numbers of cows involved is much smaller. It is worthy of note that from the 2 years' survey of the College herd no correlation could be observed either between the cell content and season of the year or between the cell content and stage of lactation, other than the well-known higher cell content found with colostrum and late-lactation milk.

Where an investigation of a low S.N.F. content in the milk of a herd is necessary, as a rule analyses of individual cow samples are essential. The results of our investigations indicate that a cell-count test, which could be done readily, could give valuable information as to a *possible* cause of the low S.N.F. content. In interpreting the results of cell counts, consideration must be given to the stage of lactation, since in very early and late lactation milk a higher cell count due to epithelial cells may be obtained. In a cell count of bulk milk a count $>100{,}000$/ml. should be regarded as suspicious. In our investigations with 163 samples (Table 2) this standard would have classified sixteen samples or 9·8 % of them as having a high cell count which was not confirmed from subsequent examination of fore-milk quarter samples. On the other hand, this standard failed to detect a small proportion (5 %) of samples where a high cell count existed in one quarter of the udder only. It should also be noted from Table 2 that, where a high cell count was found in bulk milk, generally two or more quarters of the udder were affected.

The bacteriological examination of the quarter samples from cows with high cell counts confirmed the experience of other workers, that where mastitis organisms are detected staphylococcal infections now form the great majority of cases, and that in a large proportion of cows no specific mastitis-producing organisms can be isolated.

SUMMARY

Tests over a 2-year period of milk from an Ayrshire herd showed that there was a greater incidence of low S.N.F. samples from milk with a high cell count compared with samples from that of a low cell count.

While a wide variation in S.N.F. was found at any particular cell count level, there was a broad correlation between cell count and S.N.F., the average S.N.F. for cows in the same lactation being lower for those animals with high cell counts in the milk.

It is suggested that a cell count $>100{,}000$/ml. in bulk milk should be regarded as significant.

Bacteriological examination of the high cell count samples showed that in a large proportion of cases no typical mastitis-producing organism could be isolated.

The authors wish to thank their colleagues in the Bacteriology, Chemistry and Veterinary Investigation Departments for assistance in this work.

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695. ON THE COMPOSITION OF THE PHOSPHOLIPIDS OF COWS' MILK

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(With 1 Figure)

INTRODUCTION

A wide range of values has been recorded in the literature for the phospholipid content of cows' milk (for a recent survey see Baliga & Basu (1)), but most of the more recent work indicates a value of the order of 0·03% of the wet weight. Difficulties attendant upon the extraction and purification of this minor but important fraction of the milk lipids may account for the surprising lack of data on its composition.

Baliga & Basu (2), in 1955, were the first to apply modern analytical methods to the estimation of the major components of the phospholipids of dairy products, using adsorption on magnesia to remove the 'kephalin', and differential hydrolysis with alkali to estimate sphingomyelin in the presence of lecithin. Analyses of the phospholipids of cream, butter, ghee and buttermilk were recorded (3), and the effects of species, breed, season and stage of lactation were studied (1). The composition of whole cows' milk phospholipids was given as lecithin 30%, sphingomyelin 25% and kephalin (by difference) 45%; the results for cream were very similar.

Although the method used by the Indian workers was well suited to their purpose of surveying the effects of many variables on the proportions of the major constituent phospholipids, it would not be adequate for a more detailed analysis, since large volumes of solvents would be required for the recovery of the milk phospholipids on even a modest scale, and the adsorbed kephalins are lost in the chromatographic method used.

The present study was undertaken to elucidate further the composition of the phospholipids of milk, especially of the kephalin fraction, using the chromatographic methods developed in this laboratory for the examination of the phospholipids of egg yolk (4, 5).

Since preliminary trials indicated that extraction of the total lipid of lyophilized whole milk solids followed by separation of the phospholipids from the large excess of triglyceride fat gave unsatisfactory results, it was decided to utilize the normal butter-making process as a means of rejecting most of the unwanted constituents of the milk.

The phospholipids of milk form part of the membrane surrounding the fat globules (6) and, on separation, about 70% of the total phospholipid remains with the cream (7). Most of this (*ca.* 45%) passes into the aqueous phase (buttermilk) when the emulsion is destroyed on churning (8). As the volume of the buttermilk can be as little as 2% of that of the original milk and contains only small amounts of protein and sugar it provides a convenient concentrate of part of the milk phospholipids. The remainder of the cream phospholipids (*ca.* 25% of the total) can be recovered from the butter serum obtained by

centrifuging the melted fat. Since butter serum consists of buttermilk mechanically retained among the fat granules, the phospholipid it contains would not be expected to differ from that of the buttermilk. However, the considerable proportion of the total phospholipid (*ca.* 30%) remaining with the skim-milk is presumably derived from the membranes of the smallest fat globules which escape centrifugal separation, and from microsomes or lipoprotein particles in the milk plasma (9); it might, therefore, differ in composition from the phospholipids segregated with the cream.

With these considerations in view a preliminary comparison of the compositions of the phospholipid fractions present in butter, buttermilk and skim-milk prepared from the same bulk of fresh milk was undertaken, before utilizing buttermilk as the most convenient source of a sufficient quantity of phospholipids for a more detailed examination.

EXPERIMENTAL

Materials and methods

Morning milk from the Friesian herd of the National Institute for Research in Dairying, Shinfield, was processed by conventional methods in the experimental dairy.

The analytical and chromatographic methods used in the examination of the phospholipids have been described previously (4, 10). All results are expressed on a molecular or atomic basis, usually related to the lipid phosphorus. The composition of solvent mixtures is given by ratio of volumes taken.

Table 1. *Extraction of the phospholipids by methanol–chloroform* (1:1) *from lyophilized solids of whole and dialysed milk*

Conditions	Lipid P (as % recovered)	
	Whole milk solids	Dialysed milk solids
2 hr. room temperature	85	96
Followed by 2 hr. reflux	15	4
Total phospholipid recovered (as % (w/v) of whole milk)	0·0272	0·0274

EXTRACTION OF THE LIPIDS FROM WHOLE MILK SOLIDS

Whole milk, with or without removal of the lactose by dialysis for 48 hr. against running tap water, was lyophilized and the solids treated with methanol–chloroform (1:1) at room temperature. The results (Table 1) showed that extraction of the lipid P was unsatisfactory unless the lactose was first removed.

The phospholipids obtained in this way were contaminated by the whole of the neutral fat. An attempt was made, therefore, to extract this separately by a preliminary treatment of the milk solids with acetone. However, when lyophilized whole milk solids containing 27 g. total fat were treated twice at 0° C. with 200 ml. acetone, only 13% of the lipids was removed, and this contained 14% of the total lipid P.

These unsatisfactory results, combined with the practical difficulty of lyophilizing large volumes of milk, led to the abandonment of this approach to the preparation of the phospholipids.

COMPARISON OF THE PHOSPHOLIPIDS OF BUTTERMILK, BUTTER AND SKIM-MILK

Processing

The milk (84 lb.) was treated in a commercial separator at 30° C. and the cream washed twice with an equal volume of water, the washings being added to the skim-milk. The cream was then churned and the buttermilk run off. The butter granules and the churn were washed twice and the washings added to the buttermilk.

The skim-milk was warmed to 34° C. and agitated vigorously, while 0·25 N hydrochloric acid was added until the pH was reduced to 4·6. The precipitated casein (carrying down the lipids) was allowed to settle, the supernatant fluid filtered through muslin and the curd collected and drained in a cheese press. This method of recovering the solids of the skim-milk was used in preference to evaporation of the large volume of liquid to avoid possible damage to the phospholipids during precondensation and drying.

The damp curd and the buttermilk were lyophilized, the former giving a white crumb which reduced to a fine powder in a blender, and the latter a hygroscopic fluffy brown solid which rapidly became sticky on handling.

Extraction of the lipids

Buttermilk

The buttermilk solids were powdered in a blender and shaken with 2 l. methanol-chloroform (1:1) at 0° C. for 30 min. After centrifuging, the residue was resuspended in 1 l. of the same solvent and shaken for a further 1 hr. The solvent was removed from the combined extracts at below 40° C. in a rotary vacuum evaporator and the residue taken up in chloroform, some insoluble material being rejected.

Butter

The butter was melted and centrifuged at 50° C. and the resulting serum lyophilized. The clear fat layer contained no phosphorus and was discarded; a cloudy layer at the interface was retained.

The lipids of the dried serum were recovered by extraction at room temperature with two 100 ml. portions of methanol–chloroform (1:1).

The cloudy fat layer was similarly treated with 500 ml. of solvent and the extracts combined with those of the serum solids.

Skim-milk

The dried curd was treated twice for 1 hr. with 3 l. methanol–chloroform (1:1), and twice more with 1 l. portions of the solvent. The suspensions were filtered through sintered glass, and the combined extracts evaporated to dryness and taken up in chloroform. A considerable quantity of insoluble proteinaceous material which remained was rejected.

Examination of the lipids

When the crude lipids, extracted from the three products, were dissolved in chloroform cloudy solutions were obtained indicating the continued presence of non-lipid material. Water-soluble impurities were, therefore, removed by passage through columns of cellulose powder in chloroform–ethanol–water (80:20:2) solution (11). The recoveries of P from these columns were about 90%.

The purified lipids were separated chromatographically on alumina columns using methanol–chloroform (1:1) to elute the choline-containing phospholipids (and any neutral fat) and ethanol–chloroform–water (5:2:2) to recover the adsorbed kephalins (4). The choline-containing fractions were then adsorbed on to silicic acid from chloroform solution and the neutral fat eluted with the same solvent, the phospholipids being subsequently recovered in methanol–chloroform (7:3). The recoveries of lipid P from these columns were not less than 98%.

When the three extracts were treated in this way 55% of the lipid P recovered was in the choline-containing fraction in each case, and 45% in the kephalin fraction. When examined by silicic acid-impregnated paper chromatography (10) the three kephalin fractions showed only single spots (R_F 0·8) which reacted with both the ninhydrin and the phosphomolybdic acid-stannous chloride reagents. The choline-containing fractions showed no ninhydrin-reacting material, but gave two spots staining with phosphomolybdic acid at positions corresponding to phosphatidylcholine and sphingomyelin. Lysocompounds were not detected. Other analytical data are given in Table 2.

Table 2. *Analysis of phospholipid fractions of milk products after separation on alumina columns*

	Buttermilk	Butter	Skim-milk
Choline-containing fraction			
Ratio N/P	1·25	1·32	1·54
Ratio carboxylic esters/P	1·54	1·06	1·23
Unsaturation (double bonds/P)	1·29	1·21	1·16
Kephalin fraction*			
Ratio carboxylic esters/P	2·04	1·83	2·13
Ratio inositol/P	0·006†	0·007	0·001
Unsaturation (double bonds/P)	2·30	2·51	2·37

* Serine and ethanolamine were identified chromatographically in hydrolysates in each case.
† Larger amounts of inositide were recovered from the silicic acid columns.

These results revealed no great differences between the compositions of the phospholipids of buttermilk, butter and skim-milk. The proportions of the choline-containing and kephalin fractions were identical in each case, and their mean unsaturations indicated no gross differences in component fatty acids. The N/P ratios of the choline-containing fractions were greater than unity, after removal of water-soluble contaminants, suggesting the presence of diamino-phospholipid, and the low ester/P ratios and the chromatographic results confirmed the presence of sphingomyelin.

The kephalin fractions all contained lipid-bound amino-acids and inositide, but their ester/P ratios of approximately 2·0 indicated an essentially diacylphospholipid composition.

Although quantitative recovery of the phospholipids of the three milk products was not achieved in these experiments, the compositions of the fractions isolated were so close that it was concluded that the phospholipids of buttermilk can be taken, without serious error, as representative of those of whole milk.

THE PHOSPHOLIPIDS OF BUTTERMILK

Extraction

The cream, separated from 316 lb. of milk, was washed, cooled, churned and the buttermilk lyophilized. The dried solids were extracted in two aliquots, each with 1 l. methanol–chloroform (1:1) at room temperature for 1 hr., the suspension being agitated

throughout by a stream of nitrogen. After filtration the residue was again extracted in the same way for 2½ hr. and finally refluxed with fresh solvent for 1 hr. The yields of lipid phosphorus are given in Table 3.

Table 3. *Extraction of buttermilk phospholipids by consecutive treatments with methanol–chloroform* (1:1)

Treatment	Lipid P (mg.)	Lipid P (As % recovered)
1 hr. room temperature	165	86·4
2½ hr. room temperature	22·1	11·5
1 hr. reflux	4·1	2·1

The phospholipid content (calculated as P × 25) of the total lipid recovered from the buttermilk was 8·4%. The neutral fat was eliminated by chromatography on a silicic acid column in chloroform–methanol (98:2), during which a dark green coloured band was also rejected. 9·5 gm. of purified phospholipid were recovered from the column, corresponding to 29% of that present in the whole milk taken. The main losses in the preparation were due to cream left on the surface of the cooler, and to buttermilk entrapped in the butter granules. As neither of these factors was likely to have been selective with respect to the phospholipids, the product was considered to be representative of the whole material.

Separation of the constituent phospholipids

When chromatographed on a silicic acid column in methanol–chloroform (3:7) the kephalins were recovered in a single fast-running band (Fig. 1), as had previously been found with egg phospholipids. The lecithin was next recovered in a typically elongated peak, leaving the remainder of the adsorbed choline-containing phospholipids to be eluted subsequently with methanol–chloroform–water (70:25:5).

Examination of the kephalin fraction

In two runs 47 and 48% of the total lipid P was recovered in the kephalin peak. Passage of this material through a cellulose column removed ninhydrin-reacting material which remained stationary on a paper chromatogram (11), without loss of phosphorus. The purified material gave an N/P ratio of 1·12, and repetition of the cellulose column treatment did not reduce the N/P ratio further. Analysis then showed N/P, 1·11; carboxylic esters/P, 2·05; unsaturation (double bonds/P), 2·30. Measurement of the amino N content by the ninhydrin reaction of the intact phospholipids using egg phosphatidylethanolamine as standard (12), gave amino N/P, 0·86, and estimation of the bases by the fluoro-dinitrobenzene method (13) after acid hydrolysis gave ethanolamine/P, 0·61, and amino-acid N/P, 0·20. Two-dimensional chromatography of the bases in *n*-butanol-acetic acid-water and phenol–ammonia–water gave two spots reacting strongly with ninhydrin, inseparable from ethanolamine and serine, and two other very much weaker spots which were not identified.

The plasmalogen content of the kephalin fraction was measured by reaction with *p*-nitrophenylhydrazine (14) and a molar ratio of aldehyde/P of 0·016 found. Inositol was

determined after acid hydrolysis and showed inositol/P 0·11. No attempt was made to investigate the structures of these minor components more fully.

No trace of lysocompounds could be found on the impregnated paper chromatograms.

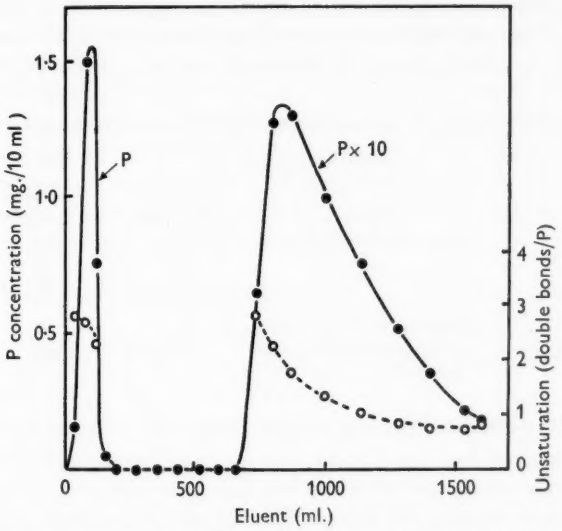

Fig. 1. Chromatogram of buttermilk phospholipids on silicic acid in methanol–chloroform (3:7), showing fractionation according to unsaturation of the kephalins (first peak) and of the phosphatidylcholine (second peak). Lipid P applied 18·7 mg., 40 g. adsorbent, column 18 cm. long, 2·5 cm. diameter.

Examination of the choline-containing phospholipids

30% of the lipid P applied was recovered in the lecithin peak which exhibited fractionation according to unsaturation, as previously found with egg lipids (15); in the present case the maximum unsaturation in the early fractions was 2·82 double bonds/P, decreasing to 0·85 at the tail of the peak (Fig. 1).

This lecithin fraction was grossly contaminated with non-phospholipid nitrogen, ratios of N/P as high as 2·99 being found in early fractions decreasing later almost to unity. The major contaminant was identified by hydrolysis and reaction with urease as urea, which appeared to travel with lecithin on the columns.

To check this point, the behaviour of a mixture of egg phosphatidylcholine and urea was investigated under various conditions. The results (Table 4) showed that urea is quite strongly bound to lecithin in methanol–chloroform solution, and that it appears toward the front of the lecithin peak when chromatographed on silicic acid in this solvent mixture. However, treatment with powdered cellulose, as normally used for the removal of water-soluble contaminants, eliminated it almost completely.

The whole of the lecithin peak, after purification by cellulose gave, on analysis, N/P, 1·08; esters/P, 1·81; unsaturation (double bonds/P), 1·24; amino N/P, 0·00. No sphingomyelin could be found by the differential hydrolysis method of Schmidt, Benotti, Hershman & Thannhauser (17), although the high N/P and low ester/P ratios suggested the presence of about 8%. The fraction was, therefore, practically pure phosphatidylcholine.

A positive reaction with *p*-nitrophenylhydrazine indicated the presence of about 5% of plasmalogen in the choline-containing phospholipids, but no reducing sugars could be detected after hydrolysis with dilute sulphuric acid, indicating the absence of cerebrosides (16).

The phospholipids remaining on the column after removal of the lecithin peak were recovered by elution with methanol–chloroform–water (70:25:5), and, after passing through a cellulose column, gave the following analysis: N/P, 1·85; esters/P, 0·57; sphingomyelin/P, 0·85; unsaturation (double bonds/P), 1·33; amino N/P, 0·00. The sphingomyelin content was found to be 85% by the differential hydrolysis method, in agreement with the proportion by which the N/P ratio exceeded unity. Since no lyso-compounds could be detected by chromatography on impregnated paper, the observed ester/P ratio indicated the presence of 28% of phosphatidylcholine, but the estimate by this method is regarded as less reliable than the other data, and the proportion of lecithin in this fraction was taken as 15% by difference.

Table 4. *Chromatography of a urea-phosphatidylcholine mixture on various adsorbents*

	Lipid P applied 39 mg.		Initial N/P ratio 2·05	
Adsorbent	Solvent	Volume of eluate (ml.)	P recovered (as % applied)	N/P ratio
Silicic acid (30 g.)	Methanol–chloroform (3:7)	250	46	3·01
		250	37	1·66
		250	13	1·05
		250	5	1·00
Alumina (45) g.)	Methanol–chloroform (1:1)	350	98	2·00
Cellulose powder (50 g.)	Chloroform–ethanol–water (80:20:2)	500	101	1·05

Table 5. *Constituents of buttermilk phospholipids*

	Moles % of total lipid P
Kephalin fraction	48
Phosphatidylethanolamine	29
Phosphatidylserine	10
Plasmalogen	0·8
Inositol	5
Choline-containing fraction	52
Phosphatidylcholine	33
Sphingomyelin	19
Plasmalogen	2·5

The results of the several analyses calculated to the basis of the initial phospholipid P are collected in Table 5.

THE FATTY ACID STRUCTURE OF THE BUTTERMILK PHOSPHOLIPIDS

Phospholipase A has been shown to hydrolyse only the α′-linkage of phosphatidylcholine, yielding the corresponding β-lysocompound and the fatty acid from the α′-position (18). These products are conveniently separated if the reaction is carried out in ethereal solution as described by Hanahan, Rodbell & Turner (19), the insoluble lysocompound being precipitated as formed and the released fatty acid remaining in solution.

This technique was applied to kephalin and phosphatidylcholine fractions prepared from the buttermilk phospholipids, and the degree of unsaturation of the fatty acids from the α'- and β-positions measured.

Phosphatidylcholine

Snake venom (*Askitrodon piscivorus piscivorus*) was used as a source of phospholipase *A*, and the reaction proceeded readily with the formation of a compact white precipitate. After 4 hr. the lysolecithin formed was centrifuged down and the supernatant solution allowed to stand overnight, after which a further precipitate was collected separately. The final ether solution was virtually free of lipid P, indicating that hydrolysis of the diacylphospholipid had gone to completion, and chromatography of the two lysolecithin fractions on impregnated paper (10) showed no trace of the faster moving initial material. The analyses of the products are given in Table 6.

Table 6. *The action of snake venom phospholipase A on milk phosphatidylcholine*

Fraction	Lipid P (mg)	Unsaturation (μ moles)	Recovery (as % initial)		Unsaturation (double bonds) /per mole)
			Lipid P	Unsaturation	
Initial phosphatidylcholine	4·47	200·3	—	—	1·39*
Ether-insoluble I	2·59	72·7	58·0	36·3	0·87
Ether-insoluble II	1·72	52·1	38·5	26·0	0·94
Recovery as lysocompound	4·31	124·8	96·5	62·3	0·90
Ether-soluble	0·07	79·4	1·6	39·6	0·55
Total recovery	4·38	204·2	98·1	101·9	—

* The lecithin molecule contains two fatty acids, the other substances one.

The kephalin fraction

The buttermilk kephalin was treated with the enzyme in the same way, but with the addition of 1 equivalent of ammonia (20). In this case a precipitate developed after about 20 min., but later redissolved. After allowing the reaction to proceed overnight only a small proportion of the lipid P was recovered in the precipitate obtained by centrifugation. However, chromatography of the lipids remaining in solution in the ether showed that the ninhydrin-reacting spot of the initial phospholipid (running at R_F, 0·80) had disappeared completely, and was replaced by a spot of equal intensity (R_F, 0·40) typical of a lysocompound. The ether-soluble products were separated by adsorption of the lysophospholipid from methanol–chloroform (2:98) solution on to a small column of silicic acid, the free fatty acid being eluted with ten column volumes of the same solvent, and the phospholipid recovered by elution with methanol–chloroform (7:3). Over 98% of the initial lipid P and unsaturation was recovered in the two fractions from the column.

Table 7. *Unsaturation of buttermilk phospholipids and of lysocompounds obtained from them*

	Unsaturation (double bonds/molecule)			
	Kephalin preparation		Phosphatidylcholine preparation*	
	1	2	1	2
Diacylphospholipid	2·24	2·56	1·70	1·39
Lysophospholipid	1·22	1·09	0·84	0·90

* These fractions were taken from the front of the lecithin peak. Their unsaturations are therefore rather higher than those given on p. 65 and in Table 2.

The results (Table 7) of experiments on two preparations of buttermilk kephalin and on two phosphatidylcholine fractions of differing initial unsaturation, showed that the fatty acids of the β-lysocompounds obtained from both phospholipids contained considerable proportions of the unsaturation present in the intact diacyl substances.

DISCUSSION

The estimate of the total kephalins obtained in the present work by chromatographic methods agrees fairly closely with that obtained by Baliga & Basu (1). The presence of several components was, however, revealed in this fraction by the examination of its hydrolysis products. The free amino bases, as estimated by the fluorodinitrobenzene method or the direct ninhydrin method accounted for nitrogen equivalent to 81 or 86% of the lipid P, respectively, approximately three-quarters being ethanolamine and most of the remainder serine. The residual kephalin P was of the same order as the lipid-bound inositol content, and may have been present in mono- or di-phosphoinositides such as have been reported previously in animal tissues (21, 22, 23).

The total nitrogen of the kephalin fraction exceeded the lipid P by 11% leaving a considerable proportion unaccounted for. No spots corresponding to any of the bases examined on paper chromatograms by Levene & Chargaff (24) could be detected in hydrolysates of these kephalins, and choline was absent. The unidentified nitrogen resisted treatment on a cellulose column, which removes most water-soluble impurities.

The choline-containing fraction of the phospholipids of milk appeared to be a mixture of phosphatidylcholine and sphingomyelin, with a small proportion of the glycerophospholipid present as plasmalogen. The sphingomyelin isolated in the present work was in rather smaller amount than that reported by Baliga & Basu (1). Lysoglycerophospholipids appeared to be absent.

The fatty acid structure of the milk glycerophospholipids differed notably from that previously found for the lecithins of egg (25) and of animal liver (26), and of egg phosphatidylethanolamine (5), in that the fatty acids in both the α′- and β-positions contained double bonds. In the other cases quoted the α′-acid has been shown to be completely unsaturated and the β-acid almost entirely saturated. The purified milk phospholipids were found to be much more readily emulsified by simple shaking than were those of egg, and it may well be that the presence of unsaturated acids in both positions in a glycerophospholipid molecule confers enhanced surface-active properties as compared with the α′-unsaturated β-saturated structure.

SUMMARY

1. The phospholipids of skim-milk, buttermilk and butter prepared from one bulk of Friesian milk were examined, using chromatographic methods. No major differences were found between the three products.

2. The phospholipids of buttermilk contained (in moles %) phosphatidylethanolamine 29, phosphatidylserine 10, phosphatidylcholine 33, sphingomyelin 19. The content of lipid-bound inositol was 5 moles % and of plasmalogens 3 moles %. Nitrogen, associated with the kephalin fraction, equivalent to 14 moles % of the lipid P was not identified. No lysoglycerophospholipids were present.

3. Unsaturated fatty acids were present in both the α′- and β-positions of the milk

glycerophospholipids, in contrast to those from other animal sources which have usually been found to contain unsaturated acids in the α'-position and predominantly saturated acids in the β-position.

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696. THE CHEMICAL COMPOSITION OF THE COLOSTRUM AND MILK OF THE EWE

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INTRODUCTION

When investigating the energetics and feed requirements of farm animals, a knowledge of the chemical composition of the colostrum and milk at various stages of lactation is important. Studies on the yield and composition of ewe milk and its relationship to the growth of the lamb have been made by a number of overseas workers (1–5). Most of this work has been on breeds different from those used in New Zealand and has not included detailed analyses of all constituents, including minerals, over the course of lactation. The comprehensive milk secretion studies of Barnicoat and his co-workers (6,7) with New Zealand Romney ewes did not report the effect of stage of lactation in association with plane of nutrition on all milk constituents. The present work gives analyses of ewe milks obtained during studies of the nutritional requirements of the ewe and lamb under different dietary levels similar to those encountered on typical New Zealand fat-lamb farms.

EXPERIMENTAL

Two groups of six 5-year-old pregnant ewes, due to lamb at about the same time, were used in this experiment. From mating time they were all grazed together under typical New Zealand store conditions until approximately 6 weeks before lambing. The high-plane animals were then provided with pasture *ad lib.*, whereas the other animals continued on a hard-grazing regime throughout the lactation. This imposed on them a somewhat low level of nutrition and they are referred to as the low-plane group. The ewes were accustomed to being handled since measurements of the individual feed intakes were made in every second week. Milk samples were taken in the alternate weeks in which no intake measurements were made.

The early colostrum samples were obtained by hand-milking, using progressively longer sampling intervals. Once the milk flow became established representative samples were taken at fortnightly intervals. On account of the difficulty of emptying the udder completely by hand-milking and thus obtaining a representative sample, it was found preferable to allow the lamb to suckle one half while the milk was withdrawn from the other. Because these animals were used to frequent handling, the milk sample could usually be withdrawn by hand in about the same time as was taken by the lamb.

The methods used for the determination of total solids, fat, protein, and ash, were those previously described for the analysis of sow's milk (8). For colostrum, smaller samples were used for total solids and protein analyses. For some of the early samples which were of small volume, the fat determination was made on an accurately weighed quantity of about 2–4 g.

Lactose was determined by a modification of the volumetric copper reduction method of Lane & Eynon (9), the milk being clarified by 10% lead acetate and saturated sodium fluoride solutions.

Chloride content of the ash was estimated by titration with silver nitrate after low-temperature incineration.

For calcium, magnesium and phosphorus the ash (from 10 g. milk) was dissolved in N/10 hydrochloric acid and analysed as described previously (10). A portion of the same acid solution was used for the determination of sodium and potassium by spectrographic analysis.

RESULTS AND DISCUSSION

Stage of lactation

The results for the major organic and mineral constituents of the colostrum and milk of six ewes in high-plane and low-plane groups are given in Tables 1 and 2. The initial secretions were characterized by high protein and low lactose values. Unlike the sow, but like most other species, the ewe yields an early secretion with a high fat content. The secretions of all the ewes showed similar trends in composition from colostrum at parturition through the transition to milk. These changes appeared to take place at slightly different rates for the various constituents, and thereafter any particular constituent altered much more slowly as lactation became established. Mean values for all samples analysed at each period in lactation are listed, while the figure in brackets gives the number of samples at each period. The following trends are apparent.

Fat. In the high-plane group, the mean fat content of the colostrum fell from the initial value of over 17% to just over 10% on the third day. The lowest fat contents of the milk were observed during the third month, after which the fat value steadily increased to over 13% at the end of the lactation. In the low-plane group there were similar trends in fat content, but the changes were less distinct and the average fat value was lower. The maximum value (14%) occurred soon after parturition, and the minimum (6·2%) at the end of the second month.

Protein. The protein contents were similar for both groups, falling rapidly from 18 to 20% at parturition to 5% during the second month of lactation, and thereafter increasing steadily to reach 8% after about 4 months.

Lactose. Lactose values were also similar for both groups, colostrum at parturition containing 1·5–2·5% lactose. As the milk flow became established, the lactose contents rose steadily until the third week (5–6%) and then decreased to 3% at the end of the lactation.

Minerals. The ash content of the colostrum of the low-plane ewes at parturition was 1·0%, falling steadily after about 12 hr. In the high-plane group the ash content was lower initially (0·8%) and then increased. These trends were apparent in all animals examined. Subsequently both groups showed the same trends throughout lactation with a minimum value (0·8%) during the third week, when the protein content was near its lowest, and the lactose near its highest value. This was followed by a steady rise to near 1·0% at the end of the lactation.

Effect of plane of nutrition

Although the trends in milk composition were similar, there were some definite differences in milk composition between the high-plane and low-plane groups. The colostrum of the low-plane group had lower fat and protein contents and higher lactose and total mineral contents than the corresponding colostrum of the high-plane group. Once the

Table 1. *Effect of stage of lactation on composition of ewe's milk (high-plane group) (expressed as percentage by weight)*

Time in milk		Fat	Total solids	Protein	Lactose	Ash	Na	K	Ca	Mg	P	Cl
(hr.)												
0–3	(3)*	17·2	39·0	19·5	1·6	0·82	0·037	0·108	0·147	0·019	0·174	0·118
3–6	(3)	15·4	33·6	15·0	2·4	0·81	0·042	0·121	0·145	0·019	0·177	0·122
6–12	(2)	14·0	30·6	12·5	3·5	0·84	0·040	0·138	0·145	0·020	0·178	0·128
12–24	(6)	12·3	28·6	11·8	3·5	0·87	0·036	0·140	0·153	0·018	0·181	0·133
24–28	(4)	10·7	22·5	7·1	4·0	0·92	0·037	0·165	0·156	0·015	0·179	0·135
(days)												
3–6	(3)	10·5	22·4	6·8	4·3	0·85	0·037	0·136	0·187	0·011	0·172	0·138
7–27	(4)	10·7	22·8	5·5	5·8	0·83	0·030	0·123	0·184	0·012	0·150	0·124
28–48		—	—	—	—	—	—	—	—	—	—	—
49–69	(6)	9·0	21·3	5·3	5·2	0·86	0·035	0·134	0·191	0·014	0·157	0·122
70–90	(6)	8·2	20·4	7·6	3·6	0·99	0·063	0·134	0·200	0·020	0·153	0·125
91–111	(12)	13·3	25·2	8·0	2·9	0·98	0·052	0·115	0·215	0·021	0·163	0·130
Colostrum—mean values (0–6 days)	(21)	13·0	28·9	11·8	3·3	0·86	0·038	0·137	0·156	0·017	0·177	0·130
Milk—mean values (7–111 days)	(28)	10·9	23·0	7·0	4·0	0·94	0·048	0·124	0·202	0·018	0·158	0·126
Milk—mean values (whole lactation)		11·8	25·5	9·1	3·7	0·90	0·043	0·130	0·183	0·018	0·166	0·128

* Number of samples analysed is shown in brackets.

Table 2. *Effect of stage of lactation on composition of ewe's milk (low-plane group) (expressed as percentage by weight)*

Time in milk		Fat	Total solids	Protein	Lactose	Ash	Na	K	Ca	Mg	P	Cl
(hr.)												
0–3	(2)*	14·0	36·9	19·4	2·5	1·00	0·048	0·115	0·181	0·025	0·230	0·074
3–6	(4)	10·9	30·3	16·0	2·5	1·01	0·047	0·138	0·196	0·020	0·210	0·133
6–12	(5)	13·0	30·0	13·3	2·9	0·99	0·042	0·140	0·194	0·019	0·200	0·142
12–24	(9)	13·2	28·0	10·5	3·4	0·94	0·038	0·139	0·171	0·017	0·198	0·144
24–28	(9)	12·7	25·4	8·4	3·5	0·92	0·038	0·160	0·179	0·015	0·195	0·137
(days)												
3–6	(6)	10·5	22·1	6·2	4·4	0·91	0·038	0·174	0·199	0·015	0·224	0·138
7–27	(3)	7·2	18·7	5·4	5·3	0·80	0·030	0·099	0·164	0·013	0·143	0·133
28–48	(8)	6·2	17·3	5·7	4·7	0·83	0·032	0·117	0·190	0·016	0·161	0·119
49–69	(6)	8·6	20·0	5·8	4·7	0·86	0·036	0·135	0·198	0·018	0·160	0·120
70–90	(6)	8·9	20·6	6·9	3·8	0·92	0·040	0·130	0·189	0·021	0·163	0·128
91–111	(9)	12·7	24·2	7·8	2·8	0·95	0·046	0·115	0·204	0·023	0·167	0·133
Colostrum—mean values (0–6 days)	(35)	12·4	27·4	10·8	3·4	0·95	0·040	0·149	0·185	0·017	0·205	0·136
Milk—mean values (7–111 days)	(32)	9·1	20·5	6·5	4·1	0·88	0·038	0·121	0·193	0·019	0·161	0·126
Milk—mean values (whole lactation)		10·8	24·1	8·7	3·7	0·92	0·039	0·135	0·189	0·018	0·184	0·131

* Number of samples analysed is shown in brackets.

milk flow was established, the milk of the high-plane ewes was appreciably richer than that from the low-plane group. Higher average values were found for fat, protein and ash contents. Calorific values per 100 g. milk, calculated from the average composition values gave:

high plane: colostrum 203 cal., milk 158 cal.;

low plane: colostrum 192 cal., milk 139 cal.

The chloride content of the low-plane colostrum at parturition was appreciably lower than that of the high-plane group. This low chloride content was probably directly

associated with the higher lactose value and the need to maintain osmotic relationships. Similar chloride values for all the ewes were found after the transition to milk. Comparison of the individual mineral constituents of the colostrum at parturition showed that the difference in total ash between the two groups was accounted for quantitatively by higher values of sodium, potassium, calcium, magnesium, and phosphorus, in the low-plane group.

The average values for mineral composition of both groups were similar, except for slightly higher values for sodium and calcium in the milk of the high-plane ewes.

Comparison with results of other workers

The average results for most of the milk constituents obtained in this study are similar to those reported by other workers (1–7, 11). In fat content alone is there a marked difference, the mean fat contents 9·1 and 10·9% of both low- and high-plane groups, respectively, being appreciably higher than those reported by Barnicoat (6,7) for New Zealand Romney ewes and Bonsma (2) for various breeds of sheep. The fat contents of ewe's milk over the course of lactation reported in the present paper are closer to, but slightly higher than, the values given by Godden & Puddy (1) and Peirce (3,4,5).

Table 3. *Summary of recorded mineral analyses of constituents of ewe's milk (expressed as percentage by weight)*

Author	Na	K	Ca	Mg	P	Cl	Group	Stage of lactation
Abderhalden (1908)	0·064	0·080	0·175	0·009	0·129	0·130	—	—
Godden & Puddy (1935)	—	—	0·209	—	0·164	—	Low Ca ration	—
	—	—	0·210	—	0·155	—	High Ca ration	—
	0·050	0·126	0·197	—	0·163	0·118	Grazing ewes—Scotland	—
Barnicoat *et al.* (1949)	—	—	0·186	—	0·201	—	High plane	Colostrum
	—	—	0·193	—	0·205	—	Low plane	Colostrum
	—	—	0·186	—	0·162	—	High/high plane	Milk 4 months
	—	—	0·171	—	0·144	—	Low/low plane	Milk 4 months
	—	—	0·171	—	0·144	—	High/low plane	Milk 4 months
	—	—	0·193	—	0·162	—	Low/high plane	Milk 4 months
Present study	0·048	0·115	0·181	0·025	0·230	0·074	Low plane	First colostrum
	0·040	0·149	0·185	0·017	0·205	0·136	Low plane	(0–6 days)
	0·038	0·121	0·193	0·019	0·161	0·126	Low plane	Milk (7–111 days)
	0·037	0·108	0·147	0·019	0·174	0·118	High plane	First colostrum
	0·038	0·137	0·156	0·017	0·177	0·130	High plane	(0–6 days)
	0·048	0·124	0·202	0·018	0·158	0·126	High plane	Milk (7–111 days)

Very few detailed studies of the mineral composition of ewe's colostrum and milk have been reported, and in any case comparison is difficult on account of differences in stage of lactation. Results are summarized in Table 3. The values in the present study suggest that the ash of ewe's milk in New Zealand is higher in potassium, magnesium, and phosphorus, and lower in sodium than those reported by Abderhalden (11). No other analysis of ewe's milk for magnesium content has been found in the literature. As far as the other mineral constituents are concerned, the results reported here agree with the values found by Godden & Puddy (1) and by Barnicoat and his co-workers (6,7). Although, in accord with the custom of other workers, mean values of all results over the course of lactation are listed, it must be emphasized that the occurrence of marked variations due to the stage of lactation limits the value of such average figures for most practical purposes in studies on the nutrition of the lactating ewe and her lamb.

SUMMARY

1. The composition of the milk of six low-plane and six high-plane ewes has been studied. Definite lactational trends have been observed for all the major constituents.

2. For all the ewes the initial colostrum was characterized by very high protein and fat contents and by low lactose content.

3. Analyses of the mineral constituents—calcium, magnesium, sodium, potassium, phosphorus, and chloride—are reported covering the whole lactation of twelve sheep. The colostrum of the low-plane animals was higher in total mineral content than that of the high-plane animals.

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697- AN ANALYSIS OF THE PERFORMANCE OF AN ULTRA-HIGH-TEMPERATURE MILK STERILIZING PLANT

I. INTRODUCTION AND PHYSICAL MEASUREMENTS

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(With 8 Figures)

INTRODUCTION

The purpose of any pasteurizing or sterilizing plant is to bring about the heat inactivation of bacteria or bacterial spores. It is generally considered for convenience that the bactericidal effect takes place almost entirely in a holding period, during which the milk can be considered to be held at a fixed temperature for a minimum time. In practice, since the milk cannot be instantaneously heated and cooled, there is a progressively increasing lethal effect throughout the period when the milk is approaching its holding temperature, and a decreasing effect in the cooling period. There is, therefore, a considerable lethal effect above that due to the nominal holding time. This has been shown in a paper by Ball(1) in which methods of calculation developed for the canning industry are applied to the problem of high-temperature, short-time pasteurization of milk. The higher the final milk temperature, the greater will be the proportion of lethal effect due to the come-up time, until, at temperatures of the order of 135° C., the lethal effect during holding may be relatively slight.

There is one factor in practical processing which has not been considered by Ball, i.e. the distribution in the velocities of different particles of milk through the plant. For hydrodynamic reasons, all the milk cannot flow at the same rate. Some particles, which remain close to boundary surfaces throughout their passage through the plant, will travel comparatively slowly and will be subject to a relatively long heating or holding time. Other particles will travel rapidly, and be subject to a short heating or holding time. Most of the milk will take a path intermediate between these extremes. The spread of velocities about the mean will depend on the degree of turbulence in the liquid flow, whether this is induced or natural. Conditions will vary in general between the heating or cooling and the holding sections, as with a plate type heat exchanger there is much induced turbulence in the heating and cooling sections, while natural turbulence occurs in the holding section. Although temperature distribution in the plant may possibly be predictable from heat transfer data, the distributions of velocities in different parts of the plant, and hence of flow times, are subject to so many variables that experimental determination is essential.

The sections of a pasteurizing or sterilizing plant where thermal destruction of bacteria takes place, i.e. the final heating, holding and first cooling sections, are shown diagrammatically in Fig. 1. Also shown are typical distributions of temperature which might be expected, and typical distributions of the times of flow of milk through the different sections, which together determine the overall time-temperature conditions of treatment

of the milk. It is the purpose of this series of papers to develop a method of predicting the bactericidal efficiency of a particular plant from the following data: (*a*) time and temperature distributions found by experiment, and (*b*) thermal destruction data appropriate to the organisms to be killed.

The plant for which the analysis is given here was a small plate-type ultra-high-temperature sterilizer with a capacity of 200 g./hr., manufactured by The A.P.V. Company. The problem of obtaining an overall lethal effect in a plant rather than of ensuring a specified minimum effect in a holding section is more difficult for an ultra-high-temperature plant than for a conventional H.T.S.T. pasteurizer, necessitating detailed analysis. If similar measurements are made, the results can be applied to any type of continuous plant, whether pasteurizer or sterilizer.

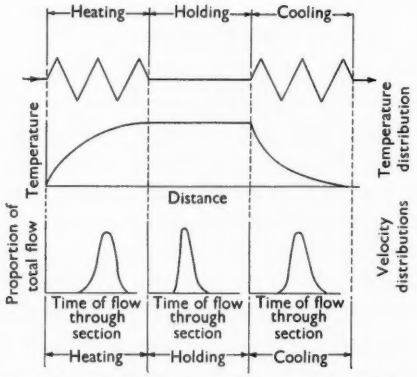


Fig. 1. Hypothetical distributions of temperature and flow times.

PHYSICAL MEASUREMENTS

The arrangement of the plant on which the experiments were made is shown in Fig. 2. From the float tank, milk passed to a regenerator stage and then to a hot-water heating stage bringing the temperature up to about 85° C. for homogenization. After this process, milk was returned to the plant to be heated in a steam-heating section to the final temperature of 130–135° C.

A variable holding section was fitted, in which the mean holding time could be varied by altering the position of displacement tubes. In this work the minimum holding time only was used. After the holding section, milk passed through the regenerator section, and then through a cold-water section, leaving at about 20° C. A hand-operated diversion valve, fitted between the holding and regenerator sections, allowed milk to be diverted through a separate water-cooling section to the float tank. The rate of flow of liquid through the plant was controlled by the homogenizer, operating as a positive displacement pump. Separate manually operated valves were fitted in the forward and diverted flow lines to allow adjustment of back pressure.

The temperature of the hot-water supply for the hot-water heating section was controlled by a conventional pneumatic controller and the steam pressure, and hence the temperature, in the final heating section was manually controlled. Thermometers and pressure gauges were fitted to the plant as shown in Fig. 2.

Throughout this work the plant was operated on water rather than milk. It was considered that under the conditions of forced turbulence in the plant, the differences in operating conditions as between milk and water would be negligible.

The physical measurements comprised measurements of temperature distribution in the steam heating and regenerator sections, and measurements of the time taken for water to pass through the steam heating and the holding sections.

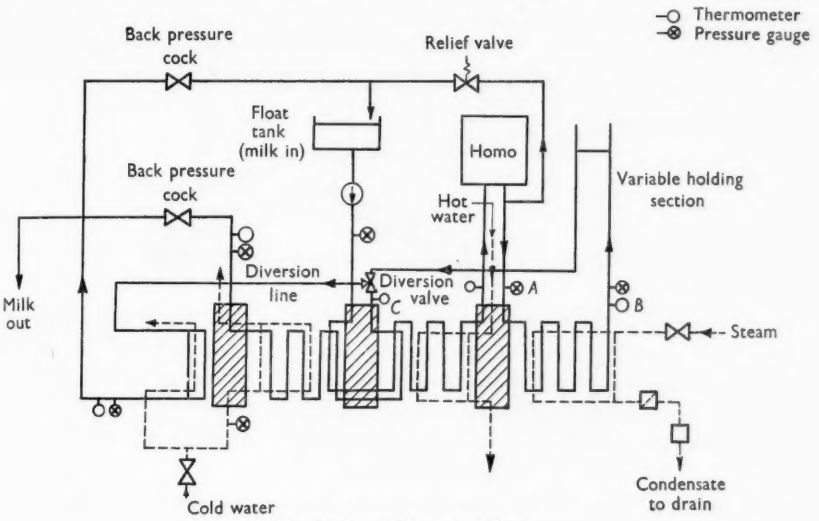


Fig. 2. General layout of plant.

While these results were being obtained, the opportunity was taken to investigate the temperature and flow conditions in the plant in more detail than was strictly necessary for an analysis of performance. As a result, it has been possible to make some observations on temperature fluctuations in the plant, and on the method of determining flow distributions by injecting soluble tracer.

Temperature measurements

Method. Copper-constantan thermocouples were used as the measuring elements. They were insulated with P.V.C., which was found to withstand the highest temperatures used unless the insulation was under stress. The thermocouples were led into the space between the plates by passing them between the gasket and the adjacent plate. The temperature and pressure at the gaskets caused the P.V.C. insulation to flatten, so that the gaskets sealed completely. At the same time, to prevent short-circuiting of the thermocouple leads at this point, they were protected on each side by a single layer of rubberized cloth. This withstood the temperatures and pressures without breaking down, and was thin enough not to disturb the gasket sealing. Initially, attempts were made to insulate the thermocouple junctions. It was found later that there was no apparent disadvantage in leaving them bare.

Twelve thermocouples were usually used. They were connected to a selector box and to a common reference junction of crushed ice in a vacuum flask. The thermocouple voltages

were measured with a vernier potentiometer. Each set of thermocouples was calibrated before use over the temperature range 90–140° C. in a thermostatically controlled oil bath against a substandard mercury-in-glass thermometer.

It was soon found that the fall in temperature in the regenerator section was so rapid that the thermal lethal effect could be considered to end with the holding section. Temperature explorations were therefore confined to the steam-heating section and the beginning of the holding section.

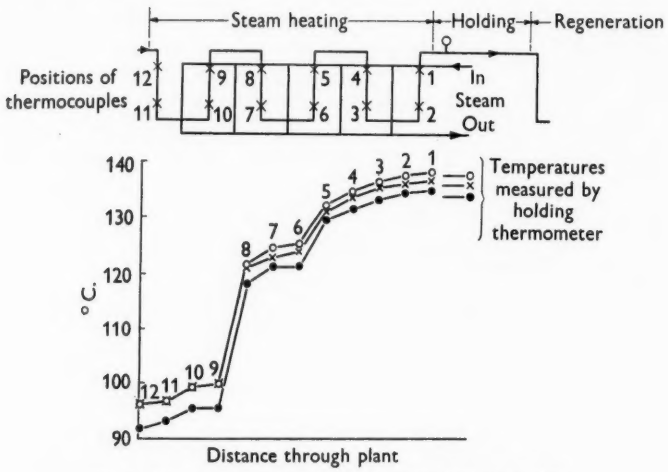


Fig. 3. Rise in temperature through steam heating section.

Results and discussion. A typical set of results, obtained for three different temperatures as measured on the thermometer at the beginning of the holding section, is shown in Fig. 3.

Consistent irregularities were obtained in the curve of temperature rise, and can be seen in Fig. 3. For example, there are particularly small temperature rises between couples 12 and 9, and between 7 and 6. The small rise for the pass between 12 and 11 is partly accounted for by the fact that this pass is heated by steam from one side only. It seems likely that the effect between 11 and 9, and between 7 and 6, is due to the accumulation of condensate in the steam section at points remote from the steam inlet, since other measurements showed comparatively low steam temperatures at corresponding points in the steam circuit. The addition of condensate and air vents at remote points in the steam circuit did not improve matters. This might have been due to the condensate being trapped in the interplate space, which has a shape designed to give high-pressure drop and induced turbulence, consequently discouraging the clearance of condensate. The vents probably took steam direct from the main steam lines and the interplate spaces not affected by condensate, and thus clearance of the affected spaces was not obtained.

To determine the short-term constancy of the temperatures obtained in the plant, records were taken of the temperature fluctuations at a point at the outlet of the last heating pass, while the steam pressure in the heating section was held constant. A thermocouple of the type described above, passed through the interplate gasket, was used together with an amplifier and high-speed pen recorder. A typical recording is shown in Fig. 4, where the absolute temperature is arbitrary.

The constancy of the temperature was good, the maximum fluctuation amounting to not more than about $\pm 0{\cdot}25°$ C. The minor variations are probably due to the high degree of turbulence in the flow, bringing water at slightly different temperatures due to the heating differential into contact with the thermocouple.

A further observation was made which has a bearing on the question of temperature fluctuations in the plant. When transparent Perspex sections of pipe were included in the flow line for observation purposes, bubbles of vapour, or gas, were seen. To eliminate these, it was necessary to apply a back pressure to the plant which was much higher than that corresponding to the temperature of operation.

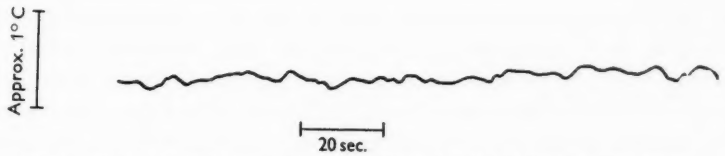


Fig. 4. Record of water temperature fluctuations at outlet of steam heating section.

For example, if the plant is operated at a temperature of 99° C., theoretically no back pressure will be required to prevent boiling. In practice, a certain back pressure is needed to prevent separation of dissolved gas, caused by rise in temperature. The bubbles of gas, if separating at any point before the end of the steam heating section, will remain at a temperature lower than that of the surrounding liquid because of their poor thermal conductivity.

Fluctuations in temperature could not be observed on recordings such as Fig. 4, when the plant was operated under conditions in which gas bubbles were produced. Probably the bubbles were of such small size relative to the thermocouple, and passed it so rapidly, that recognizable drops in temperature did not occur. At the same time, if bubbles are present they offer a possibility of spores passing through the plant under sublethal conditions, whether corresponding temperature fluctuations are experimentally detectable or not.

Experiments showed that an excess back pressure of about 15 lb./in.^{2} above that corresponding to the operating temperature was required to prevent entirely the separation of gas bubbles. The manufacturers of the ultra-high-temperature plant recommend an operating back pressure of 50 lb./in.^{2}. This corresponds to an excess of about 20 lb./in.^{2} over the pressure required to prevent boiling at an operating temperature of 135° C., so that no trouble with bubbles should be experienced. However, it is obviously important that an adequate back pressure should always be maintained in practice.

Measurement of liquid flow times

Method. The time distributions which are of importance are those for liquid flow through the heating section and through the holding section, i.e. between points *A* and *B*, and *B* and *C*, respectively on Fig. 2.

The method of obtaining flow distributions was a modification of the method of holding-time determination described by Dummett & Mongar (2), and the apparatus described by them was used. The tracer fluid was sodium nitrite, detected by the Griess–Ilosvay reagent as described by Taylor (3) and by Botham (4).

The sampling cock was fitted at a suitable socket on the plant at the end of the section for which measurements were to be made, i.e. either at the thermometer socket at *B* or at the thermometer socket at *C*. It was adjusted before each test to give a flow of liquid through the cock of 60 ml./sec. This is almost a quarter of the throughput of the plant, but no modification of the flow occurs before the sampling cock, so there is no disturbance of the flow times in the region of interest.

The injection syringe is intended for use in pasteurizing plant, and not against the high back pressures used in ultra-high-temperature sterilizers. However, injection of the tracer was completed in a small fraction of a second, and this was considered satisfactory. The syringe was filled with saturated sodium nitrite solution and was attached to the plant through a spring-loaded non-return valve at the socket either at *A* or at *B*.

At zero time, the injection syringe was operated. Samples were collected from the sampling cock in a set of ten test-tubes at times determined by preliminary experiment. Each test-tube contained the output from the sampling cock in $\frac{1}{2}$ sec. period, so that each test-tube contained approximately 30 ml. of water containing a certain concentration of sodium nitrite.

Control samples of sodium nitrite solution were prepared as follows: A stock solution of 0·1 mg. nitrite per ml. was diluted to 0·01 mg. per ml. In seven test-tubes were placed 0·125, 0·25, 0·5, 1·0, 2·0, 4·0 and 8·0 ml. of this solution, each tube being made up to 10 ml. by the addition of distilled water.

The experimental samples were serially diluted as required, by taking 1 ml. volumes into test-tubes containing 9 ml. of distilled water.

When the control series and the experimental dilutions were completed, 0·5 ml. of Griess–Ilosvay reagent was added to each tube, and the characteristic pink colour was allowed to develop. After about 10 min., each experimental dilution was ranked against the control series by visual comparison. Two persons independently made the comparisons, and an average of their findings was later taken. The comparisons were always completed in considerably less than 30 min., before fading of the colour in the most concentrated control sample had become serious.

If reference was necessary to a less dilute experimental sample, 0·45 ml. of reagent was added to the 9 ml. remaining in this tube.

Results and discussion. A typical set of results for the holding section is given in Table 1. The sampling cock was found to have a capacity of 15 ml., which at a flow rate of 60 ml./sec. gives a sampling lag of 0·25 sec. This time has been subtracted from the

Table 1. *Variation of nitrite concentration with time after injection, for holding section between points B and C*

Time of sampling after injection (sec.)	Corrected sampling time* (sec.)	Dilution of sample	Ranking against controls	Nitrite in sample (mg./10 ml.)
0·5–1·0	0·25–0·75	10^{-1}	0·125	0·125
1·0–1·5	0·75–1·25	10^{-1}	0·125	0·125
1·5–2·0	1·25–1·75	10^{-2}	1·9	19
2·0–2·5	1·75–2·25	10^{-3}	6	600
2·5–3·0	2·25–2·75	10^{-3}	7	700
3·0–3·5	2·75–3·25	10^{-3}	3·5	350
3·5–4·0	3·25–3·75	10^{-3}	1	100
4·0–4·5	3·75–4·25	10^{-3}	0·4	40
6·0–6·5	5·75–6·25	10^{-2}	0·45	4·5

* Allowing time of 0·25 sec. in sampling cock.

nominal sampling times to give the corrected times referring to the plant alone. The distribution of holding times is shown graphically in Fig. 5, which also shows a smoothed distribution curve. It can be seen that the fastest particles of liquid pass through the holding section in about 1·5 sec., but that more take about 2·5 sec. than any other time.

When short times of this order are being measured, the efficiency of the injection and sampling processes is of considerable importance. The correction noted above for the sampling cock probably leaves little further error at this point. To examine the injection process for possible sources of time lags, transparent plastic sections were incorporated in the pipework at the injection point and concentrated methylene-blue solution was injected from the syringe.

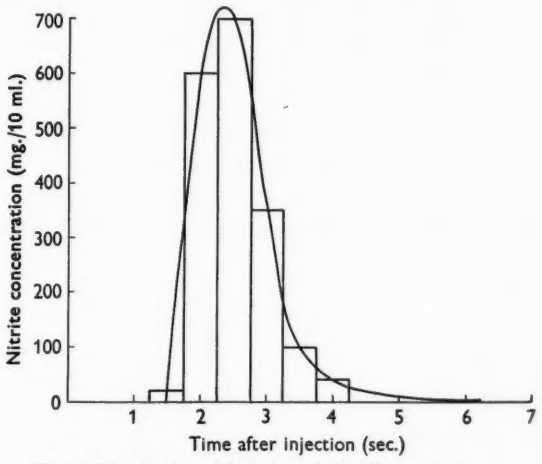
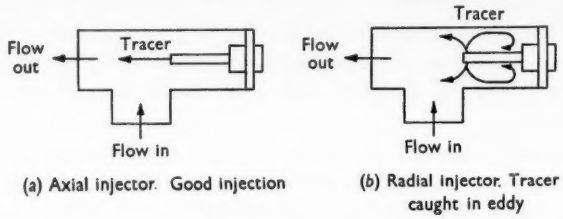


Fig. 5. Distribution of flow times for holding section.

Two types of injection nozzle were supplied with the testing apparatus, one with a single axial hole, and one with a series of radially-directed holes. The injection point for the holding tube was at a tee-piece in the flow line, and the injection nozzle when in place was in a position relative to the flow line as shown in Fig. 6. When dye was injected using the different types of nozzle, it was found that with the axial hole the dye was immediately picked up by the flow and carried away. This situation is shown diagrammatically in Fig. 6(*a*). With the radial holes some of the dye was trapped in an eddy as shown in Fig. 6(*b*) and only leaked into the main flow comparatively slowly. As a result, different flow distributions were obtained in the two cases, as shown in Fig. 6(*c*).

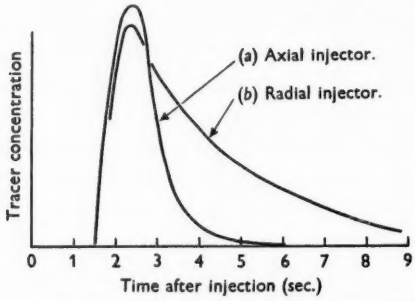
This demonstrates the importance of satisfactory injection conditions if correct flow time distributions are to be obtained. Variations of this kind have little effect on the determination of a minimum flow time, but may be serious where the whole distribution is required, as in this case. It is understood that the axial injector is the one which is generally used, with the radial injector only used in special circumstances. Nevertheless, the geometry of the injection point will determine how good a result is obtained.

The mean holding time given by Fig. 5 is 2·5 sec. The volume of the holder, obtained experimentally, was found to be 670 ml. which, at a flow rate of 200 g./hr., corresponds to



(a) Axial injector. Good injection

(b) Radial injector. Tracer caught in eddy



(c) Distribution curves for both types of injector

Fig. 6. Effect of injection arrangement on flow distribution curve.

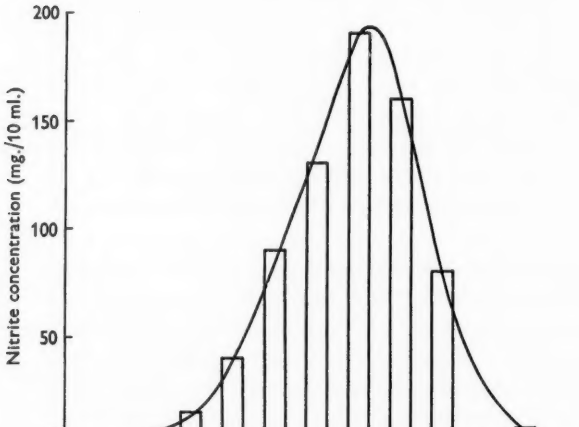


Fig. 7. Distribution of flow times for heating section.

a theoretical mean holding time of 2·56 sec. There is, therefore, little likelihood of there being further undetected injection and sampling time lags.

A typical flow distribution curve for the whole of the heating section, with the injector at *A* and the sampling cock at *B* (Fig. 2), is shown in Fig. 7. It can be seen that the distribution is much more symmetrical than that for the holding section, and in fact the experimental nitrite concentrations fit quite closely to a normal distribution curve having a mean time of 16·8 sec. and a standard deviation from the mean of 1·54 sec.

As a further examination of the performance of the heating section, it seemed of interest to see how the flow distribution curve changed for different lengths of the heating section. It was possible to remove pairs of passes from the experimental machine by removing the corresponding plates, and in this way the six-pass normal heating section could be successively reduced to four passes, two passes, and finally to only the associated pipework.

The flow distributions for these cases, corrected for a constant total nitrite content in each case, are shown together in Fig. 8. The mean flow times for the different effective holding sections are: 16·8 sec. for 6 passes, 12·45 sec. for 4 passes, 7·75sec. for 2 passes,

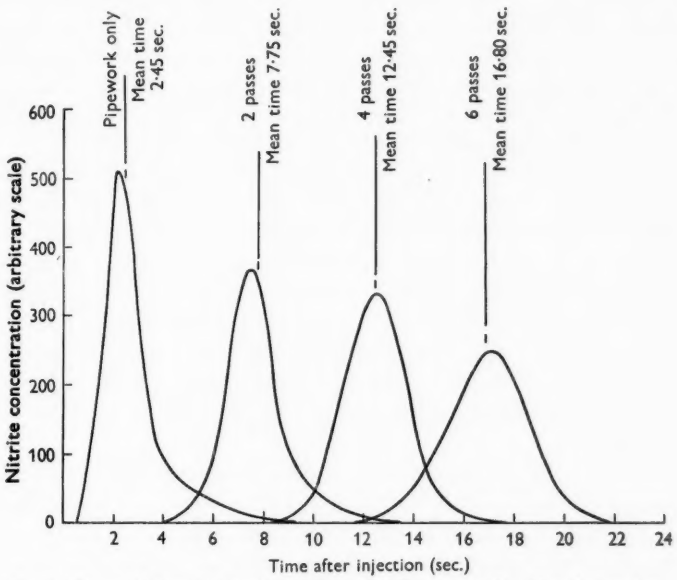


Fig. 8. Corrected flow distribution curves for different lengths of heating section.

and 2·45 sec. for the associated pipework only. The calculated mean flow time for the pipework only, obtained from its calculated volume and a flow rate of 200 g./hr., is 2·50 sec., which agrees well with the experimental value.

The differences between the mean flow times should correspond to the flow times through one pair of passes. These differences are 4·35, 4·70 and 5·30 sec., with a mean flow time per pair of passes of 4·78 sec. The measured projected area of one plate is 1·59 sq.ft., and the interplate spacing is 0·156 in. At a flow rate of 200 g./hr. this gives a mean flow time per pair of passes of 4·63 sec., which agrees well with the mean experimental figure of 4·78 sec. The differences in the times between passes were not investigated, since they are comparatively small. It is possible that they were caused by plate deflexion under pressure altering the volume of different passes.

The symmetry of the distribution curves increases with increasing numbers of passes. With four and six passes the curves are almost normal, but with smaller numbers the asymmetry is considerable, and a 'tail' develops on the curves. The distribution for the pipework alone is very similar to that for the holding section, which indicates that this shape of curve is typical for naturally turbulent flow in passages of circular cross-section.

It seems probable that a section of intense induced turbulence, such as an interplate space, introduces a Gaussian distribution of flow times, which is superimposed on any distribution which already exists.

This is in agreement with the theory of tracer distribution developed by Taylor (5) for long, straight pipes, which shows that the distribution at some distance from the point of injection is Gaussian if a high degree of turbulence exists. If the Reynolds number is so low that the laminar boundary layer contains an appreciable amount of diffusing tracer material, a long 'tail' in the concentration-time curve may disturb the symmetry. In view of the high degree of turbulence deliberately induced in the interplate spaces, a Gaussian distribution is to be expected for this part of the circuit. In the pipework a different picture is obtained. The Reynolds number for flow through a 1 in. diameter tube at 200 g./hr., taking a kinematic viscosity of 0·5 centistokes for the fluid, is only about 25,000, so the laminar layer will be fairly thick and may well account for the asymmetry in the pipework part of the heating section and in the holding section.

It is of interest to consider briefly here how well a soluble and diffusible tracer material such as sodium nitrite, nickel chloride, or common salt, reproduces the behaviour of discrete suspended particles such as bacteria or bacterial spores. The theory developed by Taylor (5) applies to a soluble tracer material. Batchelor, Binnie & Phillips (6) have extended the theory to the case of discrete particles of finite size. Where Taylor developed an effective diffusion constant K, which determines the distribution of the tracer and has a value of 10·1 in a straight pipe, Batchelor *et al.* (6) have expressed their results in terms of a diffusion constant $K(\alpha)$ which is a function of a variable α. α is the ratio of the particle diameter to the pipe diameter for flow in a circular pipe. When $\alpha = 0$, i.e. for a soluble tracer, $K(\alpha) = K = 10{\cdot}1$.

If we consider the case of a bacterium of diameter 1μ moving in the space 0·156 in. wide between the heat exchange plates, the factor α is of the order of 0·00025. This is a much smaller value than is considered by Batchelor *et al.* (6), so that $K(\alpha)$ in this case must approach the value 10·1 corresponding to a true solution. It therefore follows that the dispersion of bacteria in turbulent flow must approach that of soluble tracer, and probably there is little fundamental inaccuracy in assuming that the two are the same. At the same time, $K(\alpha)$ is varying very rapidly with α in this region, so that there must be some difference, however small, between the two cases. There is scope for further investigation of this point.

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698. EFFECT OF THE HEAT TREATMENT OF MILK ON THE YIELD, QUALITY AND CERTAIN PROPERTIES OF STANDARDIZED SEPARATED MILK CHEESE

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INTRODUCTION

Separated milk cheese is very popular in Egypt because its price falls within the limited incomes of the masses. Originally it was made from milk after skimming off the cream, the skimmed milk being left until acid coagulation occurred. The resultant cheese was of reasonably good body and texture, and of fairly palatable taste because of the nature of this type of coagulation and the amount of fat remaining in the milk after gravity separation.

Recently, because of the introduction of separators, separated milk is now replacing skim-milk for making this type of cheese, and rennet coagulation is used. As a result of this type of coagulation and the negligible amounts of fat in separated milk, the cheese obtained is usually unpalatable, tough and leathery. In order to improve the properties of the cheese it was thought desirable to standardize the separated milk to contain a small amount of fat and to pasteurize it before making it into cheese. The effect of such treatment on the yield, quality and other properties of this type of cheese is of economic importance, because considerable amounts of separated milk are used for cheese-making.

EXPERIMENTAL

Four experiments were carried out. In each experiment varying amounts of separated buffalo's milk were used, to which whole milk was added to raise the fat content to about 1·5%. The fat, total solids and total nitrogen of the milk were determined.

In each experiment the milk was salted in the proportion of 7% and then divided into two equal parts. One part was heated to renneting temperature only (90° F.), whilst the other was heated to 170° F. for 15 min. and then cooled to 90° F. Liquid rennet was added in the proportion of 25 and 33 ml. per 100 lb. of raw and pasteurized milk respectively.

At the end of the coagulation period, which took 2½ hr., curd was ladled into moulds and left to drain. When draining was complete (which required 4 days including the day of manufacture) the cheese was taken out of the moulds and placed on a muslin cloth for 2 hr. before judging and sampling.

Sampling

(*a*) *Milk.* Samples were taken for the determination of fat, solids-not-fat (S.N.F.) and total nitrogen after the standardized milk had been well stirred in the cheese vats.

(*b*) *Cheese.* After removing the moulds a representative sample of cheese was taken by cutting a sector. The sample was ground with a sterilized pestle and mortar.

(*c*) *Whey.* Whey was strained, warmed up to 90° F., well stirred and then sampled for the determination of fat, total nitrogen and acidity.

Yield of cheese

The resultant cheese in each experiment was weighed. The whey was collected all through the draining period and weighed.

Cheese grading

The raw milk and pasteurized milk cheese were judged by three persons using a scale of 100 maximum points divided into flavour (50), body (20), texture (20) and general appearance (10). The average markings of the three persons were recorded.

Shelf life

To assess the shelf-keeping property the cheese was left on the shelf and graded daily for 5 days. Room temperature ranged from 26–30° C.

Analytical methods

(*a*) *Milk.* Fat content was determined by the original Gerber method, total solids by drying 2 ml. of milk to constant weight in an oven at 100° C., and total nitrogen by the method of Rowland (1).

(*b*) *Cheese.* The British Standards methods (2) were used for determination of fat content and total solids (overnight method). Sodium chloride was determined by the wet digestion method of Davies (3), and total nitrogen by the method described by Rowland (1) for milk using 0·3 g. of sample for digestion and steam distillation and N/20 solutions.

(*c*) *Whey.* Fat was determined by the original Gerber method using special butyrometers, total nitrogen by the Rowland method (1) using diluted whey equivalent to 2 ml. whey and N/50 solutions, and acidity by titrating 10 ml. whey with N/9 NaOH and an 0·5% solution of phenolphthalein.

Bacteriological examination

Total colony counts of the cheese were determined on standard nutrient agar and on standard milk agar prepared according to Chalmers (4). Plates were counted after incubation at 30° C. for 48 hr.

RESULTS AND DISCUSSION

The quality of cheese as shown by grading

Table 1 shows the points awarded for flavour, body, texture and general appearance of both raw and pasteurized skim-milk cheese, together with the averages attained by each group in the four experiments.

The figures show that pasteurization of the milk materially improved both the flavour and the texture of the resultant cheese, but had little or no effect upon its body and general appearance.

The shelf life of fresh cheese

The average scores for flavour, body, texture and general appearance of both groups of cheeses resulting from the four experiments, when judged on 5 successive days, are given in Table 2. The average scores for flavour, body, texture and general appearance of the

cheese made from pasteurized separated milk were always higher than those of the cheese made from raw milk up to the third day.

On the fourth and fifth days, however, the average markings of the cheese made from raw milk were higher than those of the cheese made from pasteurized milk. The total markings of the former were 62·5 and 55, while those of the latter were 60 and 45·25 on the fourth and fifth days successively. This was due to defects of flavour, body, texture and

Table 1. *Grading fresh cheese from raw and pasteurized separated milk*

No. of experiment	Flavour		Body		Texture		General appearance		Total	
	R	P	R	P	R	P	R	P	R	P
(1)	35	45	16	18	12	18	6	8	69	89
(2)	35	40	15	18	10	20	7	8	67	86
(3)	35	45	17	18	17	18	6	9	75	90
(4)	40	45	18	18	15	18	9	8	82	89
Average	36·25	43·75	16·5	18	13·5	18·5	7	8·25	73·25	88·5

R = cheese made from raw separated milk.
P = cheese made from pasteurized separated milk.

Table 2. *Average scores of cheese from four experiments during storage for 5 successive days*

Storage period (days)		Flavour	Body	Texture	General appearance	Total
1	R	36·25	16·5	13·5	7·0	73·25
	P	43·75	18	18·5	8·25	88·5
2	R	32·0	15	13·5	7	67·5
	P	37·75	16	18·5	8	80·25
3	R	30·0	13·25	15	6	64·25
	P	32·5	14	19	6	71·5
4	R	29·5	12·0	15	6	62·5
	P	25	12·5	17	5·5	60
5	R	25·25	10·5	15	4·25	55
	P	20	9·25	13	3	45·25

R = cheese made from raw separated milk.
P = cheese made from pasteurized separated milk.

general appearance of the cheese made from pasteurized separated milk that appeared 3 days after manufacture. This is not in agreement with the well-known effects of pasteurization on the quality of hard cheeses. Thus, Davis (5) has stated that the destruction of bacteria and longer ripening period associated with pasteurization result in considerably increased keeping quality. In this work the cheese made from pasteurized milk became on the fourth and fifth days slimy and slippery as a result of the growth of moulds, which gave this soft type of cheese a mouldy flavour and appearance, spoiling its quality. It seems that the superiority of the pasteurized milk cheese in the early stages of its keeping on the shelves might be due to the fact that it commences with a much more restricted flora. However, the pasteurized milk cheese contained a very considerable number of organisms (Table 3), and from the analytical data given later in the paper it seems possible that this cheese might provide a slightly better growth medium, because of its higher moisture content and greater retention of various nitrogen compounds and salts giving moulds better chance to grow and thus causing its spoilage.

Yield of the cheese

Table 3 shows the yield of both raw and pasteurized milk cheese, together with the amounts of whey in each of the four experiments. In all experiments the pasteurized milk gave a higher yield of cheese. There was an average increase of 3·33% in the yield of the cheese when milk was pasteurized prior to cheese making.

Table 3. *Fat, total nitrogen* (T.N.) *and solids-not-fat* (S.N.F.) *of milk, together with the yield of cheese and whey from raw and pasteurized skim milk*

No. of experiment	Type of milk	Weight of milk (1·5% fat) in lb.	% fat in milk	% T.N. in milk	% S.N.F. in milk	Cheese		Whey	
						Weight in lb.	% of weight of milk	Weight in lb.	% of weight of milk
(1)	R	30	1·45	0·5866	9·48	7·40	24·66	22·40	74·6
	P	30	1·45	0·5866	9·48	7·80	26·00	22·00	73·3
(2)	R	24	1·45	0·6616	10·59	6·57	27·30	16·75	69·7
	P	24	1·45	0·6616	10·59	7·40	30·88	16·00	66·6
(3)	R	25	1·5	0·5656	9·35	6·10	24·40	18·00	72·0
	P	25	1·5	0·5656	9·35	7·40	29·64	16·75	67·0
(4)	R	25	1·4	0·5204	8·77	5·40	21·84	19·00	76·0
	P	25	1·4	0·5204	8·77	6·20	24·80	17·25	69·0
Average	R	26	1·45	0·5835	9·54	6·36	24·50	19·06	73·3
	P	26	1·45	0·5835	9·54	7·20	27·83	18·00	69·2

R = raw milk cheese. P = pasteurized milk cheese.

Similar increases have been reported by Rogers (6) for factory-made Neufchatel and Cottage cheese from pasteurized milk, and by Tretsven (7) for Cheddar cheese made from a mixture of equal parts of normal high temperature—short-time pasteurized milk and milk heated at 194° F. for 20 min.

The increase in yield of the separated milk cheese made from pasteurized milk over that made from raw milk could be explained by the action of pasteurization in the production of soft curd, which is capable of holding more moisture than the curd resulting from unheated milk. The weight of whey liberated from pasteurized milk curd was always less than from raw milk curd, the average difference being 4·1%.

It would appear that the increase in the yield of the cheese made from pasteurized milk is largely due to its ability to retain more moisture than the cheese made from raw milk. However, the retention of some other constituents in the pasteurized milk curd was demonstrated by chemical analyses, and these may also contribute to the increased yield.

Chemical analysis of skim-milk cheese from raw and pasteurized milk

Table 4 shows the percentages of moisture, total solids, and also the percentage of fat, total nitrogen and chlorides *per se* and as percentage dry matter in cheese made from raw and pasteurized separated milk in the four experiments, together with their averages. The same table also shows the fat, total nitrogen and acidity of whey.

Moisture and total solids. The moisture content of the cheese made from raw milk (average 70·12%) was lower than that of cheese from pasteurized milk (average 72·84%), whilst the total solids of the raw milk cheese (average 29·87%) were higher than for the pasteurized milk cheese (average 27·16%). This provides further evidence that the increased cheese yield from pasteurized milk is related to moisture retention.

Table 4. *Analysis of cheese made from raw and pasteurized milk and the resultant whey*

Experiment no.		Moisture (%)	Fat in cheese		T.S. of cheese (%)	T.N. of cheese		Sodium chloride of cheese		Fat of whey (%)	T.N. of whey (%)	Acidity of whey (%)
			Per se (%)	Per dry matter (%)		*Per se* (%)	Per dry matter (%)	*Per se* (%)	Per dry matter (%)			
(1)	R	70·11	5·85	19·57	29·89	1·776	5·94	4·89	16·36	0·01	0·1480	0·28
	P	72·67	5·4	19·38	27·33	1·649	6·02	5·15	18·84	0·01	0·1380	0·18
(2)	R	71·96	5·25	18·72	28·04	1·912	6·81	5·03	17·93	0·01	0·1372	0·20
	P	74·18	4·64	17·97	25·82	1·789	6·93	5·37	20·76	0·01	0·1218	0·14
(3)	R	69·96	6·15	20·45	30·02	1·796	5·98	4·86	16·18	0·01	0·1218	0·32
	P	73·47	4·95	18·65	26·53	1·621	6·11	5·22	19·67	0·01	0·1252	0·17
(4)	R	68·45	6·36	20·15	31·55	1·716	5·43	4·75	15·05	0·01	0·1356	0·36
	P	71·04	5·61	19·37	28·96	1·627	5·62	4·92	16·98	0·01	0·1316	0·21
Average	R	70·125	5·90	19·74	29·875	1·800	6·04	4·88	16·38	0·01	0·1372	0·29
	P	72·84	5·15	18·84	27·16	1·6715	6·115	5·17	19·04	0·01	0·1266	0·175

R = raw milk cheese.
P = pasteurized milk cheese.

T.S. = total solids.
T.N. = total nitrogen.

Fat. The fat content of raw milk cheese averaged 5·9% in the cheese and 19·72% in the dry matter, whilst that of pasteurized milk cheese averaged 5·17% and 18·82%. A higher fat content would be anticipated in the raw milk cheese because of its lower moisture content, but on a dry-matter basis both cheeses might be expected to yield the same figure. The slightly lower fat in dry-matter figure for the pasteurized milk cheese may be due to the retention of some additional solids, such as water-soluble salts and heat coagulated proteins.

Total nitrogen. The higher average nitrogen content of raw milk cheese (1·80%) than of pasteurized milk cheese (1·67%) may again be attributed to the lower moisture content of the former. On a dry-matter basis the slightly higher figure for pasteurized milk cheese (6·11 against 6·04%) may indicate some incorporation of heat-coagulated albumin and globulin from the pasteurized milk. This might also play some part in the increased yield of pasteurized milk cheese. Tretsven (7) also reported that the increased yield of Cheddar cheese obtained from pasteurized milk was accounted for by retention of heat coagulable protein.

Salt. The noticeable increase in the sodium-chloride content of the pasteurized milk cheese may also be due to greater retention of water which contains sodium chloride in solution.

The fat content of the whey was the same for both groups of cheese, and was very low, as would be expected. The total nitrogen content of the raw milk whey (average 0·137%) was higher than that of pasteurized milk whey (average 0·127%), which again suggests some retention of heat-coagulated albumin and globulin in the pasteurized milk cheese.

The much lower acidity of the whey from pasteurized milk would result from the destruction of acid-producing organisms by pasteurization. The rather low acidities of all the wheys was probably due to some inhibitory action by the added salt.

Total bacterial count of cheese

Table 5 shows the total colony count of all cheeses on both nutrient agar and milk agar.

Milk agar always gave higher counts than the nutrient agar with both raw milk and pasteurized milk cheese. The average increase for milk agar compared with nutrient agar was 44·97 and 60·7% with the raw milk and pasteurized milk cheese, respectively.

Table 5. *Total colony count of fresh cheese made from raw and pasteurized separated milk*

	Millions per gramme			
	Raw separated milk cheese		Pasteurized separated milk cheese	
Experiment no.	N.A.	M.A.	N.A.	M.A.
(1)	202	704	23	44
(2)	138	165	18	27
(3)	256	282	24	66
(4)	625	618	65	72
Average	305·25	442·25	32·5	52·25

Increase in average counts between N.A. and M.A. 137 (44·9%); 19·75 (60·7%).

N.A. = nutrient agar. M.A. = milk agar.

Pasteurization effected a considerable reduction in the bacterial population of the cheese. The calculated percentage reduction was 89·3 and 88·1% when using nutrient agar and milk agar media, respectively.

SUMMARY AND CONCLUSIONS

1. Four batches of soft cheese were made using rennet coagulation from both raw and pasteurized separated milk standardized to contain 1·5% fat and 7% added sodium chloride.

2. The yield of pasteurized milk cheese was about 3·33% higher than that of raw milk cheese.

3. Pasteurization of the milk improved the flavour and the texture of the fresh cheese, but had no marked effects on the body and general appearance.

4. The shelf keeping property after 4–5 days under the conditions of these experiments was better for the cheese made from raw milk. Reasons for this apparent anomaly are discussed.

5. Chemical analyses showed a greater moisture retention by the pasteurized milk cheese, leading to a lower content of fat and total nitrogen but increased sodium chloride in solution. Some retention of heat coagulable protein in the pasteurized milk cheese was suggested by a higher total nitrogen in dry-matter figure and by a lower nitrogen content of the pasteurized milk whey.

6. Pasteurization of the milk greatly reduced the total bacterial population of the fresh cheese and also the acidity of the whey produced.

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699. LACTOBACILLI IN CHEDDAR CHEESE

I. THE USE OF SELECTIVE MEDIA FOR ISOLATION AND OF SEROLOGICAL TYPING FOR IDENTIFICATION

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(With 6 Figures)

The lactobacilli are considered to play an important part in Cheddar cheese ripening because they increase in numbers as the flavour develops, and because they form a large part of the flora of the mature cheese (Davis (1); Sherwood (2)). It was thought that the presence of certain species or strains might be associated with the different flavours of different cheese. It is, therefore, desirable to be able to identify the lactobacilli from cheese not only as particular species, but as strains which can then be traced in their distribution throughout the cheese during the ripening period, and in other situations.

In the past, identification of lactobacilli has been confused and difficult, and it has often been impossible to reconcile the results of different workers. However, recent work on classification (Rogosa, Wiseman, Mitchell, Disraely & Beaman (3); Briggs (4); Wheater (5,6); Davis (7)) has provided a sound basis of simple biochemical tests which can be used to identify the species. In regard to subdivision into strains, Sherwood (2) found that he could distinguish 'biotypes' by their characteristic fermentation patterns, and both Rogosa *et al.* (3) and Davis (7) divided their species into a number of biochemical groups. A disadvantage of this method is that the identification of a large number of isolates (which is inevitable in studies on cheese) by many biochemical tests is a long and tedious process which in itself limits the number of isolates which can be studied. It was thought that the identification of the lactobacilli by serological typing based on the methods of Sharpe (8) would solve this problem, providing a rapid and accurate technique for the identification of the strains, and also a means of tracing the organisms occurring in several different cheeses. Sharpe (9) has already shown that known types of lactobacilli inoculated into cheese milk can be recovered from the cheese and identified by this method.

Before the evolution of selective media for lactobacilli it was impossible to isolate these organisms in the early stages of cheese ripening, when the starter streptococci were dominant, and it was assumed that lactobacilli did not begin to grow until the streptococci had begun to die out. Mabbitt & Zielinska (10), using a modified medium of Rogosa, Mitchell & Wiseman (11) which inhibited the growth of the starter streptococci, showed that the lactobacilli grew slowly from the beginning of the ripening period. They did not characterize their isolates beyond the generic level and the types of lactobacilli occurring at this stage still await investigation. This medium (10) was found by Naylor (12) to be particularly favourable for the growth of lactobacilli such as *Lactobacillus casei*, *L. plantarum* and *L. brevis* which commonly occur in cheese.

The object of this work was to study a Cheddar cheese to find the numbers and types of lactic acid organisms occurring and their distribution throughout the ripening period.

In particular, the medium of Mabbitt & Zielinska (10) was to be tested in comparison with media commonly used for such work, i.e. yeast dextrose agar and tomato dextrose agar, to assess its value for selectively isolating lactobacilli from cheese. At the same time the use of serological typing as a means of identifying the lactobacilli was to be investigated.

METHODS

A 40 lb. cheese, designated L2, was made from raw milk by the normal Cheddar process in the Institute's experimental dairy. It was ripened at 55° F. Two starters of known phage type (712 and 924) were used.

At each sampling of the cheese three cores were taken and the holes filled with sterile paraffin wax. The top $\frac{1}{2}$ in. of the core was removed and the remainder chopped into $\frac{1}{4}$ in. thick slices. An 8–9 g. sample was chosen at random and weighed into a sterile flask. 80 ml. of 2% sodium citrate solution at 50° C. was added and the sample homogenized in a blender for 3 min. The homogenate was allowed to stand 15 min. before plating.

Isolation of lactobacilli and streptococci

Suitable dilutions of the homogenates from each core were plated in duplicate in yeast dextrose agar (YDA), tomato dextrose agar (TDA) and acetate agar (AcA). The AcA was made according to the method of Mabbitt & Zielinska (10). The TDA was based on the medium of Briggs (13) and contained dextrose, 2%; Evans peptone, 1·5%; sodium chloride, 0·5%; Yeastrel, 0·3%; Tween 80, 0·01%; agar, 2% (all w/v) and tomato juice, 10%. The medium had a final pH of 6·6. The YDA contained Evans peptone, 1%; Lemco, 1%; sodium chloride, 0·5%; dextrose, 0·5%, Yeastrel, 0·3%; agar, 2% (all w/v) and had a final pH of 7·0.

Plates were incubated anaerobically in an atmosphere of 90% H_2 + 10% CO_2 for 5 days at 30° C. Preliminary experiments showed that anaerobic was better than aerobic incubation for the growth of lactobacilli. After counting, twenty colonies from each medium were picked into broth. Colonies from YDA were picked into dextrose Lemco broth (DLB), from TDA into tomato dextrose broth (TDB) and from AcA into acetate broth (AcB). All cultures were purified by streaking on TDA or YDA and picking single colonies.

Identification of cultures

Cultures were Gram-stained, and tested for catalase production by the addition of 0·5 ml. 20 vol. H_2O_2 to 1·5 ml. of a broth culture.

Streptococci. The streptococci were phage typed as follows. A 1% inoculum of a litmus milk culture was added to three tubes of litmus milk, one serving as a control, and to each of the others were added two drops of the appropriate phage preparation. These were incubated overnight at 30° C. when the lysed strain failed to clot and to reduce the litmus milk. When 'slow' strains were isolated they were tested on plates. YDA plates were dried for 2 hr. at 37° C., then swabbed with YDB cultures of the streptococcus strain. Drops of phage were added when the plates were dry and these were incubated overnight at 30° C., when cleared areas showed the lysed strain. In both types of tests controls of known phage types were included.

Lactobacilli. All Gram-positive, catalase-negative bacilli or coccobacilli were included in this group.

Serological typing

A number of strains with different biochemical reactions were selected and type sera prepared using the method of Sharpe (8) for making lactobacillus group antisera, except that formol killed and not heat killed suspensions were used. HCl extracts (Sharpe (9)) of all lactobacilli isolated were tested against these sera by ring precipitin tests; some of the organisms which failed to react were used to prepare further type sera, and this procedure continued until most of the isolates were typed.

Absorption tests. Any cross-reactions occurring between different type sera were removed by absorption with the heterologous reacting strains, using 1 vol. of packed cells to 4 vol. of serum. It was sometimes necessary to absorb the sera more than once or to absorb with more than one organism, to obtain a specific serum.

Biochemical tests were then carried out on each of the type strains to identify them fully. To establish that the serological types were uniform biochemically as well as serologically, representative strains of each type which had been isolated from different cores in the cheese at different times during the ripening period were selected and tested, using a smaller number of biochemical tests which were characteristic for the type strains.

Biochemical tests

The inoculum for all tests except gas from glucose was 0·05 ml. of a vigorously growing culture in TDB. Incubation was at 30° C. unless otherwise stated.

The methods described by Briggs (4) were used for the tests for growth at 15° C. and 45° C. and production of NH_3 from arginine. Those of Wheater (5,6) were followed for growth in yeast dextrose litmus milk and for fermentation of carbohydrates.

Production of gas from glucose. The method of Gibson & Abd-el-Malek (14) was used, the inoculum being the deposit of a centrifuged 10 ml. TDB culture. This large inoculum was found more satisfactory for *L. brevis* strains which otherwise often gave false negative results because of their very slow growth and consequent slow gas production.

Growth in the presence of sodium chloride and Teepol. Screw cap bottles of TDB containing different concentrations of Teepol or sodium chloride were inoculated and observed for growth. The Teepol was found more reliable than the bile salts used by Wheater (5); 0·1% Teepol corresponding to 4% bile. The homofermentative lactobacilli were incubated in this medium for 2 days and the heterofermentative lactobacilli for 1 week.

Hydrolysis of aesculin. Aesculin tomato broth containing Evans peptone, 1·5%; NaCl, 0·5%; Yeastrel, 0·3%; aesculin, 0·2%; ferric ammonium citrate, 0·5% and Tween 80, 0·01% (all w/v) and tomato juice 10% (v/v) at pH 6·6 was used. The inoculated medium was observed for 1 week for blackening.

Hydrolysis of sodium hippurate. The organisms were grown for 14 days in TDB + 1% sodium hippurate, then 1·5 ml. 50% H_2SO_4 were added to 1 ml. of the supernatant, giving white crystals of benzoic acid in positive reactions.

RESULTS

The counts on any one medium for the three separate cores taken at any one time were always very similar, the extremes never varying from the average of the three by a factor of more than two. Hence, the average of the results from the three cores has been taken at each sampling.

Comparisons of the total counts on the three media throughout the ripening period (Fig. 1), and the numbers of lactobacilli and streptococci occurring on these media (Fig. 2) show that the initially high counts on TDA and YDA were due to streptococci, which diminished and finally disappeared. At about 50 days the numbers of lactobacilli rose above the streptococci and only lactobacilli were isolated on all three media later in the ripening period.

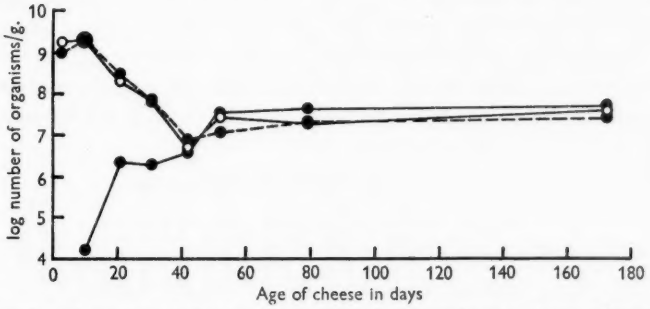

Fig. 1. Total number of organisms isolated on three media from samples of a cheese, taken throughout the ripening period. ●----●, total count on yeast dextrose agar; ○——○, total count on tomato dextrose agar; ●——●, total count on acetate agar.

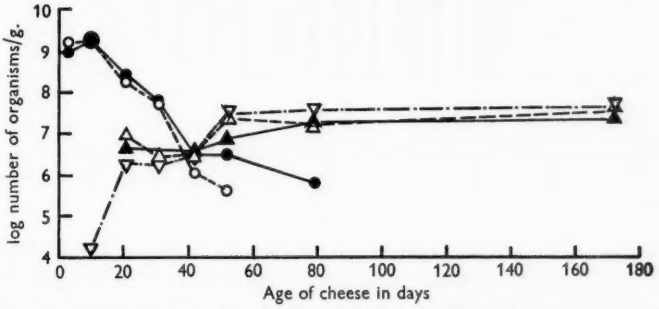

Fig. 2. Numbers of streptococci and lactobacilli isolated on three different media from samples of a cheese, taken throughout the ripening period. ●——● streptococci on yeast dextrose agar; ○----○, streptococci on tomato dextrose agar; ▲——▲, lactobacilli on yeast dextrose agar; △----△, lactobacilli on tomato dextrose agar; ▽——▽, lactobacilli on acetate agar.

Fig. 3 shows in percentages the relative proportions of lactobacilli, streptococci and miscellaneous organisms among twenty colonies isolated from each core on each medium during the ripening period. It can be seen that the AcA was the most efficient medium for the isolation of lactobacilli as they could be isolated on it very early in the ripening period, whereas on the other media large numbers were not isolated until 42 days. Only nine miscellaneous organisms were isolated during the whole of the ripening period: four were *Bacillus* spp., two were pediococci, one a *Leuconostoc*, one a *Micrococcus* and one a group D *Streptococcus*.

Lactobacilli isolated

Of the 855 lactobacilli isolated 841 were subdivided into seventeen serological types by means of precipitin reactions. These consisted of three types of *L. brevis*, six of *L. plantarum*

and eight types of low-temperature homofermentative lactobacilli which were designated 'the unclassified group'.

Tests with the *Lactobacillus* group sera of Sharpe (8), renamed by Sharpe & Wheater (15), confirmed that all the *L. brevis* strains reacted with the group E serum, four out of six of the *L. plantarum* strains reacted with the *Lactobacillus* group D serum, but none of the unclassified group reacted with antisera to either *L. casei* or *L. plantarum*.

The fourteen isolates which were not typed serologically were tested biochemically and found to consist of four distinct biochemical types. Five strains were *L. plantarum* (type strain J 48); five fell into the unclassified group (AA 2); three were *L. brevis* (AA 25) and one (T 37) was different from all the other strains and fell into the unclassified group. These biotypes have been included with the serological types in the following data.

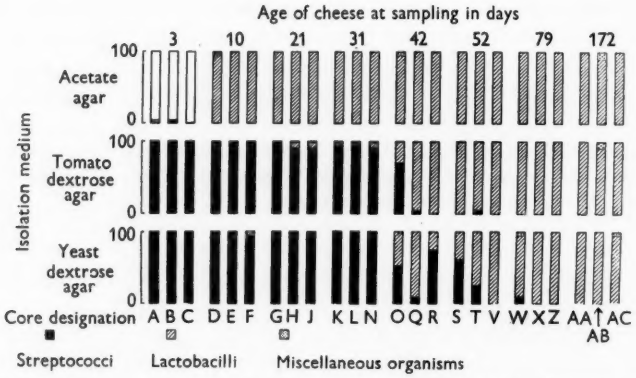

Fig. 3. Comparison of the percentages of lactobacilli, starter streptococci and miscellaneous organisms isolated on three different media from each core at each sampling of cheese. Approx. 20 colonies identified from each sample.

The biochemical reactions of all the types are shown in Table 1. The *L. brevis* types fell into group VII of Briggs (13) and showed the typical reactions of *L. brevis* in producing gas from glucose, NH_3 from arginine, and growing at 15° C. but not 45° C. Usually they fermented arabinose, xylose, dextrose and galactose but did not ferment many other sugars.

The seven *L. plantarum* types had the reactions described by Wheater (6) and by Rogosa *et al.* (3).

The remaining types (V 4, AC 10, K 44, A 41, D 45, V 5, T 58, T 6, AA 2 and T 37) fell into group VI of Briggs (13) but not into any well-recognized species. V 4 and AC 10 appeared to form a group apart from the other unclassified strains. In general their reactions were intermediate between those of *L. casei* and *L. plantarum* but they were not closely related to either. They resembled both *L. casei* and *L. plantarum* in hydrolysing aesculin and fermenting amgydalin, cellobiose, melezitose and salicin. However, they did not ferment mannitol which is almost invariably fermented by these species. Like *L. casei* they did not ferment melibiose, raffinose, or rhamnose, but like *L. plantarum* they formed a slow acid clot in yeast dextrose litmus milk. V 4 was distinguished from AC 10 by failing to ferment maltose.

The other unclassified strains were not homogeneous but had certain characters in

Table 1. *Biochemical reactions of type strains and representatives of the type strains isolated from cheese* L2

Lactobacillus group	...	I. *L. brevis*	II. *L. plantarum*	III. Unclassified

Table 1. *Biochemical reactions of type strains and representatives of the type strains isolated from cheese* L2

Lactobacillus group	...	I. *L. brevis*				II. *L. plantarum*							III. Unclassified								IV	
Type strain	...	V7	T21	K46	AA25	L50	H10	W60	AB41	O55	K50	J48	K44	A41	D45	V5	T58	T6	AA2	T37	V4	AC10
No. of strains tested in selected tests		9	2	2	3	5	4	5	2	5	5	5	13	12	6	7	5	2	5	1	3	5
Growth at 15° C.*		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 45° C.*		−	−	−	−	−	±	−	±	+	−	−	−	±	−	−	−	−	−	−	−	−
Gas from glucose*		+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
NH_3 from arginine*		+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Growth in 0·1% Teepol		±	+	+	+	+	+	+	+	+	+	+	−	−	−	±	−	−	±	−	+	+
Growth in NaCl 4%		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in NaCl 6%		+(±)	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in NaCl 8%		−	−	−	−	+	+	+	−	+	−	+	−	−	−	−	−	±	−	−	−	−
Growth in YDLM		Very slow A	Slow ACR	Very slow AC	Very slow AC	Med ACR	Med ACR	Med ACR	Med ACR	Med ACR	Med ACR	Slow ACR	Slow ACR	Slow ACR	Med ACR	Slow ACR	Slow ACR	Slow ACR	Slow A	Slow AC	Slow ACR	Slow ACR
Hydrolysis of aesculin (II, III, IV)		−	−	±	±	+	+	+	+	+	+	+	−	−	−	±	−	−	±	−	+	+
Hydrolysis of hippurate		−	+	+	+	+	+	+	−	+	−	+	−	−	−	−	−	+	−	+	±	−
Production of acid from:																						
Amygdalin		−	−	−	−	+	+	+	+	+	+	+	+	+	+	±	+	−	−	±	+	+
Arabinose (I, II, IV)		+	+	+	+(−)	+	±	+	+	+	+	+	−	−	−	−	±	±	+	−	+	+
Cellobiose (II, III, IV)		−	−	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
Dextrin		−	−	−	−	+	−	±	+	±	±	±	−	−	−	−	−	−	−	−	−	−
Dextrose		+	±	+	+(−)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol		−	−	−	−	−	−	−	−	+	−	−	−	−	−	±	−	−	−	−	−	−
Galactose		±	±	+	+(±)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+
Glycerol		−	−	−	−	−	−	±	±	+	−	±	−	±	−	±	+	+	−	−	−	−
Lactose*		±	±	+	−	+	+	+	+	+	+	+	−	+	+	+	−	+	+	−	+	−(+)
Laevulose (I)		±	±	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose (II, IV)		+	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
Mannitol (II, III, IV)		−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
Mannose		−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Melezitose*		−	±	−	−	+	+	−	+	+	+	+	−(±)	±	±	±	−	−	−	−	+	+
Melibiose*		−	±	−	−	+	+	+	+	+	+	+	−	−	+	+	−	−	−	−	−	−
Raffinose (II, III, IV)		−	−	−	−	+	−(±)	+	+	+	±	±	−	−	+	+	−	−	−	−	−	−
Rhamnose (III)		−	−	−	−	−	+	−	+	+	−	±	+	+	+	±	−	−	−	−	−	−
Salicin		−	−	−	−	+	+	+	+	+	+	+	−	−	−	+	−	−	±	−	+	+
Sorbitol (III)		−	−	−	−	+	+	+	+	+	+	−	±	−	−(±)	+	±	+	−	−	−	−
Sorbose (III)		−	−	−	−	−	−	−	−	+	−	−	±	−(±)	−	−	−	+	−	−	−	−
Sucrose		−	−	−	−	+	+	−	+	+	+	+	+	+	+	±	+	−	−	+	+	+(−)
Trehalose (II, IV)		−	−	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	+	±
Xylose (I)		+	+	+	+(±)	−	−	−	−	+	−	−	−	−	−	±	−	−	−	−	−	−

Notes.

±=slight, late or variable reaction.

All strains failed to ferment glycogen, inositol, inulin and starch.

In YDLM Slow=more than 4 days to produce action; Med=3 or 4 days to produce action; A=acid; C=clot; R=reduction.

This table shows the reactions of the type strains. Representatives of the type strains were also tested in a selected number of tests which are shown as follows:

* Representative strains in all groups tested. I, II, III and IV, representative strains in the appropriate group only tested. Figures in parenthesis refer to differences in reaction from the type strain given by representatives of the strain.

common. They were all coccobacilli, usually pear-shaped, and all fermented dextrose, galactose, laevulose and maltose. All were either aesculin-negative or showed variable reactions. This was in contrast to *L. casei* and *L. plantarum* as well as V4 and AC10 which were aesculin positive. All failed to ferment two or more of the typical *L. casei* and *L. plantarum* sugars, i.e. amygdalin, cellobiose, melezitose, mannitol, salicin and trehalose. All except D45 and V5 failed to ferment melibiose and raffinose, the typical *L. plantarum* sugars. Thus, all strains were relatively inactive in sugars and their reactions were unlike those of the named species of low-temperature homofermentative lactobacilli. Cheeseman, Berridge, Mattick & Bottazzi (16) using chromatographic methods showed that V4 and AC10 were closely related to each other, but not closely related to the other unclassified types. Their chromatographic patterns fell almost halfway between those of *L. plantarum* and *L. casei*, having some of the characters of each species. The rest of the unclassified types, except A41 which closely resembled *L. plantarum*, were related to *L. plantarum* but did not have the typical picture.

The biochemical tests on representative strains of each serotype isolated at different times during the ripening period, showed that these strains were essentially the same as the type strain. Minor variations did occur, generally in the ability to ferment a single sugar, but in all basic characteristics the strains were identical. The variations which occurred are shown in the brackets in Table 1.

An unusual variation occurred in type AC10 when one strain, J60, which fell into this type, fermented lactose but failed to ferment sucrose—the opposite to AC10 and other strains belonging to this type. A serum was prepared against J60 and reciprocal absorptions showed that it was identical with AC10. This showed that some biochemical variation could occur within a serological type. With this exception the biochemical reactions of strains falling into the same serological type were so consistent that the conception of the types as subdivisions within each species was felt to be justified.

Comparison of the three isolation media

AcA was found neither to inhibit nor to favour any of the serological types. All the types characterized, except T37, were isolated on AcA as well as on the other media, showing that they were all capable of growth on it. T37 of which only one strain occurred was isolated on YDA but grew quite well on AcA. The fact that all the types occurring were isolated on either one or both of the usual isolation media YDA and TDA as well as on AcA, indicated that the AcA was not favouring any particular type. V7 strains were isolated infrequently on YDA, although 133 were isolated altogether, and did not grow well when streaked on this medium. Thus, if only YDA had been used for an isolation medium V7 would be assumed not to be present in large numbers. Many more lactobacilli were isolated on AcA than on YDA or TDA, because they were isolated on it early in the ripening period when streptococci were being isolated on the other media.

Distribution of lactobacilli during ripening

Fig. 4 shows the relative numbers and distribution of the types at each sampling time, combining all isolates on the three media from the three cores, whilst Fig. 5 shows the distribution of the cultures isolated on AcA only. These two figures are almost identical

up to 42 days because the only lactobacilli isolated were those on AcA. In the later samples the isolations from YDA and TDA are included in Fig. 4 and this may give a misleading appearance. For instance, in this figure the numbers of K44, and to a lesser extent A41 and D45, appeared to increase markedly in the later samples. This was because these organisms were isolated on YDA which did not support the growth of type V7, the predominant type at this stage on TDA and AcA. Thus, Fig. 5 gives a truer picture of events, as it compares equal numbers of isolates at each sampling although the total numbers are less. Except for this difference the results in both cases are similar.

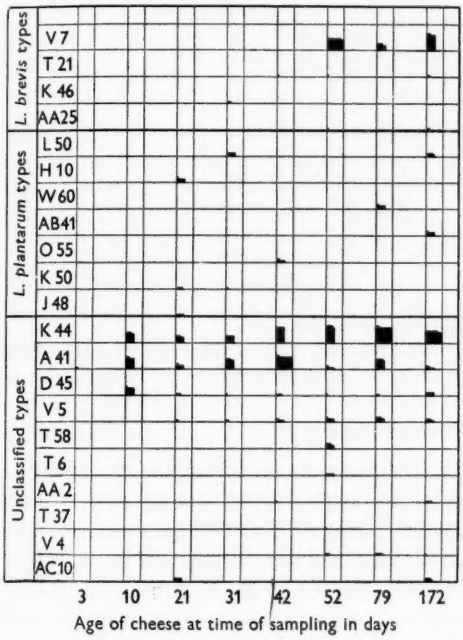

Fig. 4. Distribution throughout the ripening period of all lactobacilli isolated from the cheese. —, represents five organisms.

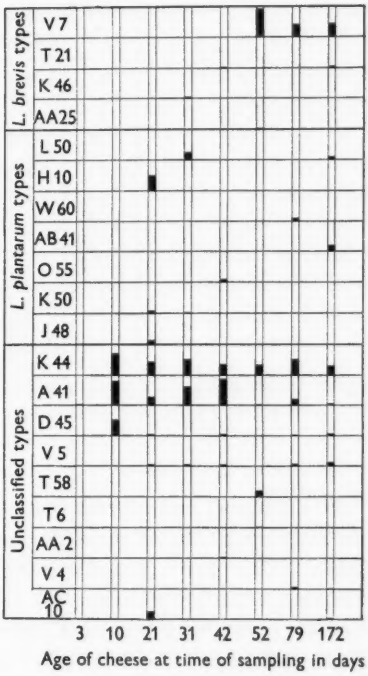

Fig. 5. Distribution throughout the ripening period of the lactobacilli isolated on acetate agar from the cheese. —, represents five organisms.

The whole picture shows a basic flora of a few types which are present throughout the ripening period, supplemented by isolated groups of organisms appearing at random in the samples. At 52 days the basic flora was partly replaced by a heterofermentative type which persisted until the end of the sampling.

Distribution of organisms in cores taken at the same time

Fig. 6 illustrates the numbers and distribution of the types of lactobacilli and the starter streptococci isolated on three media from the cores S, T and V taken when the cheese was sampled at 52 days. This illustration was deliberately selected as it shows the greatest variability in all the samples taken.

K 44 and V7 were isolated from all cores but not on all media. All the other types were isolated from only one or two of the cores showing on a smaller scale the occurrence of 'pockets' of organisms as well as the basic flora.

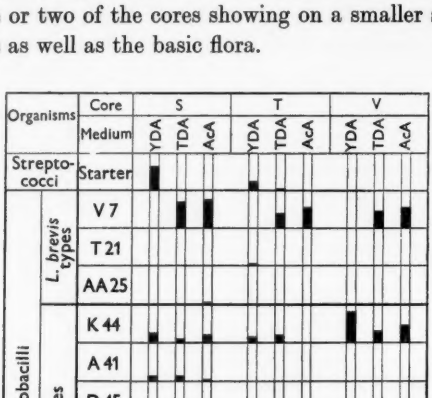

Fig. 6. Distribution of the organisms isolated on three media from three cores of the cheese at 52 days. YDA, yeast dextrose agar; TDA, tomato dextrose agar; AcA, acetate agar; ■, represents five organisms.

The starter streptococci

Five hundred and fifty-six starter streptococci were isolated from the cheese during the ripening period. Of these 551 were *Streptococcus lactis* (712) and five were *Str. cremoris* (924). A few 'slow' strains of both types were isolated. Thus, 712 almost completely dominated 924. Tests of the whey at cheese-making showed no evidence of phage. Cross-spotting of the strains on YDA plates showed no inhibition of 924 by 712, and the reason for the predominance of 712 is not known.

DISCUSSION

The success of the AcA as an isolation medium was abundantly demonstrated. Of the 440 strains isolated from it only five were not lactobacilli. Only one of these was a starter *Streptococcus* and as this occurred on the plate with undiluted homogenate containing more than 10^8 starter organisms/ml., the efficacy of the acetate as an inhibitor of starter is remarkable.

Lactobacilli were isolated on AcA without difficulty in the early stages of cheese ripening. Previous workers had been unable to isolate lactobacilli until the streptococci began to decline, and this was so with the isolations on TDA or YDA. The use of AcA gives a new insight into the rise of the lactobacilli which occurs slowly from the very beginning of the ripening period.

AcB is useful in ensuring that colonies of lactobacilli picked from AcA will not be outgrown by other organisms which may be present in large numbers as a background in

the AcA plates. In addition the lactobacilli isolated, particularly *L. brevis* strains, grew better and more rapidly in AcB than in TDB, facilitating successful isolation from the original plates. Growth of V 7 and K46 in TDB took as long as 5 days, while these cultures grew in 1–2 days in AcB. Without AcB many of these strains would not have been isolated.

No type selection of lactobacilli on the acetate media was observed. All the types which were isolated on YDA and TDA were also isolated on AcA and, within the limits of experimental error, in the same proportions. With V 7, which grew slowly on TDA, YDA did not support growth adequately, and only a few isolations were made from this medium. However, the numbers appearing on TDA were similar to those on AcA, so that the AcA was giving a true picture of the *Lactobacillus* flora. Thus AcA, combined with AcB, proved a satisfactory means of isolating lactobacilli from cheese.

Serological typing proved a successful method of identifying lactobacilli isolated from the cheese. Of the 855 lactobacilli isolated, 841 were subdivided into seventeen serological types, and it is likely that most of the remaining fourteen could also have been typed if sera had been prepared. Some of these latter organisms (J 48 type), however, did not appear to produce type antigens, but their similar pattern of antigenic cross-reactions made them a distinct serological entity. The serological method had several advantages: the subdivision of the species was greater than that shown by the biochemical tests, and when large numbers of organisms of the same type were isolated it gave a true picture of their relative numbers within each species; once sera were prepared large numbers of isolates could be rapidly and certainly identified, particularly when absorbed specific sera were used; and in the group of unclassified organisms which has not been defined biochemically, serological typing showed that at least eight subspecies were present.

The validity of the serological types was well demonstrated by the uniformity of the biochemical reactions of different strains of the same type isolated at different times throughout the ripening period. The case of J 60 and AC 10 which were identical by reciprocal absorption, but had two differing sugar fermentations, was the only instance of a marked difference in reactions occurring in all the strains tested. This shows that it is injudicious to define a variety on a difference of a single sugar fermentation.

No evidence was obtained of any of the strains becoming weakened as described by Davis (1), as the fermenting abilities of strains isolated late in the ripening period were identical with those isolated early, within any serological type. No evidence of any loss of type antigen, such as might be shown by weakening of the serological type reaction, of strains isolated late in the ripening period was observed; although some strains with weak type reaction did occasionally occur, their distribution was spasmodic. Absorption tests always showed that they were identical with the type strain. In some cases type sera were prepared from more than one strain within the type and these were always identical by reciprocal absorption.

The shape of the growth curves for the lactobacilli and streptococci in cheese were very similar to those given by Mabbitt & Zielinska (10) and both showed the crossing of the curves when the cheese was about 6 weeks old. The numbers of lactobacilli per gramme of cheese were less in our cheese (10^7–10^8) than in theirs (10^8–10^9). Also their lactobacilli appeared to be increasing in numbers at 4 months, while in our case the numbers remained stationary. However, it is likely that total numbers as well as the rate of multiplication will vary in different cheeses, depending on the temperature of ripening as well as on the available carbon and nitrogen sources.

The dominance of the *Str. lactis* starter (712) over the *Str. cremoris* (924) was almost complete. No obvious inhibition, or phage attack was responsible and a difference in metabolic activity may have been the cause.

The occurrence of *L. plantarum* in cheese is well known (Davis (1); Sherwood (2)), but the frequency of occurrence is variable. Davis (1) found that *L. plantarum* occurred when the cheese was between 1 and 5 months old and did not exceed 20% of the lactic acid organisms, while Sherwood (2) found that 62% of his isolates from pasteurized milk cheese were *L. plantarum*, occurring at all stages in the ripening period. The results obtained here showed a similarity to those of Davis's in that *L. plantarum* represented only 8% of the lactobacilli isolated, but whereas most of his isolates were *L. casei* most of our isolates were the unclassified types.

The late appearance of *L. brevis* is also well known, although Davis (1) failed to isolate any of these organisms. If types similar to V7 were present in the cheese he examined it is unlikely that they would have been detected on his isolation medium (YDA). Sherwood (2) found that 6% of his isolates were *L. brevis*, whereas in this experiment 17% were found.

The unclassified group of low-temperature homofermentative lactobacilli formed the majority flora of this cheese, and efforts to relate them to other species described have not been successful, although descriptions are often inadequate and differences in test media may give different results. They do not appear to be weakened *L. plantarum* or *L. casei* strains of Davis (1), nor the intermediate group of Sherwood (2). They may be a uniform group containing several varieties, or may represent several species hitherto unnamed in the group of homofermentative lactobacilli, but this cannot be ascertained until more isolates from this and other sources are examined.

The results of the distribution of the various types during the ripening period showed that only a few of the twenty-one types were present in large numbers, and that there was a basic flora of two of the unclassified types, A 41 and K44, with a partial replacement by *L. brevis* type V7 in the later stages. The other types of *L. brevis*, *L. plantarum* and the unclassified strains appeared to occur in 'pockets' and were only isolated spasmodically. The use of the serological typing method was particularly useful here as it showed that the organisms in the 'pockets' were often found in only one core. The variability of the samples presents great difficulty in cheese ripening studies. The results reported here show that large variations can occur in cores taken at the same time. If 'pockets' of organisms occur, and these have been described in Stilton cheese by Hiscox, Harrison & Wolf (17), their inclusion in a sample would give false emphasis to their numbers in relation to the other flora. As there is a limit to the number of colonies which can conveniently be isolated and identified, it would seem best to pool the cores and isolate from this combined sample on the assumption that the different 'pockets' would each contribute to the total flora. An estimate of the average results from the three cores of any one sampling showed this to be promising.

SUMMARY

A comparison of three different media, yeast dextrose agar, tomato dextrose agar and acetate agar (which is selective for lactobacilli) showed that the acetate medium was satisfactory for the isolation and counting of lactobacilli from Cheddar cheese during ripening. Lactobacilli were isolated on this medium in the early stages of ripening when the large numbers of starter streptococci growing on the other media were completely

suppressed. Lactobacilli isolated were identified biochemically and serologically. Serological typing was found to be a useful method of identifying large numbers of isolates and of assessing the incidence and distribution of particular types in the cheese.

We wish to thank Dr A. T. R. Mattick for his interest and helpful advice in this work; Dr L. A. Mabbitt for preparation of the cheese samples; Miss H. R. Chapman for making the experimental cheese; and Mrs P. Jarrett for technical assistance.

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700. FACTORS INFLUENCING THE ACTIVITY OF CHEESE STARTERS. THE ROLE OF MILK PEROXIDASE

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(With 4 Figures)

In our previous paper (1) it was shown that the rising of cream plays an important part when assessing the activity of starters in pasteurized milk (161° F./15 sec.); while the acid production of certain slow starters was greatly enhanced by preventing creaming, others remained slow and only became fast when the milk used had been heated to a higher temperature. This latter type of slow starter which appears to be inhibited by a heat labile substance present in milk, forms the subject of the present work.

The effect of heat treatment of the milk in which the starter is grown has received attention from many workers, and their findings are included in a review by Babel (2). Auclair & Hirsch (3), Auclair & Berridge (4) and Auclair (5) stated that there are two heat labile substances, lactenin 1 and 2, present in milk which, acting together, are capable of inhibiting certain organisms, including a cheese starter. Jago (6), working with cheese starter cultures, found that the inhibitory substance present in both raw and pasteurized milk was inactivated by boiling and appeared to be associated with the fat globule. He suggested that it might be an enzyme. Finally, the whole field of inhibition of organisms in milk has recently been reviewed by Berridge (7).

EXPERIMENTAL

Method

As in earlier work (1) the activity of a starter was assessed by inoculating 10 ml. of a heat-treated milk with 1 ml. of 1/10th dilution of an autoclaved milk culture incubated at 22° C. for 24 hr., and measuring the acidity developed after 6 hr. storage at 30° C. by titration against N/9 NaOH.

Procedure

A few preliminary experiments showed that the slow starter 972 (*Streptococcus cremoris*) behaved quite differently from the slow starter 803, since it failed to give a good activity in homogenized high temperature short time (H.T.S.T.) pasteurized milk, and since the temperature of heating necessary to bring about a marked increase in activity appeared to be considerably above that necessary to damage seriously the creaming properties of milk. In order to clarify these differences the following experiment was set up.

Bulk commercial H.T.S.T. and homogenized H.T.S.T. milk were repasteurized to various temperatures, using a 50 gal./hr. plate heat exchanger, type H.T.A., which has an effective holding time of 23–24 sec. 10 ml. quantities of these milks were placed in sterile test-tubes, cold stored overnight and inoculated the following morning with 1% of starter 803, which agglutinates, and thus is carried up with the cream. Similar inoculations were made with starter 972. The activities (mean of duplicate tubes) are shown in Fig. 1.

Fig. 1 shows that the activity of 803 is very similar in all homogenized milks irrespective of the temperatures of reheating up to 180° F., while higher temperatures have a slightly adverse effect. Starter 972 on the other hand, is inhibited in homogenized milk, but its activity increases with the heat treatment, reaching its optimum in milk reheated to 190° F. Using pasteurized milk, 803 develops maximum activity in milk reheated to 175° F. approx., while 972 shows only a slightly increased activity (0·310) in such milk; in fact it only reaches its highest activity in milk reheated to 195° F. approx. This indicates

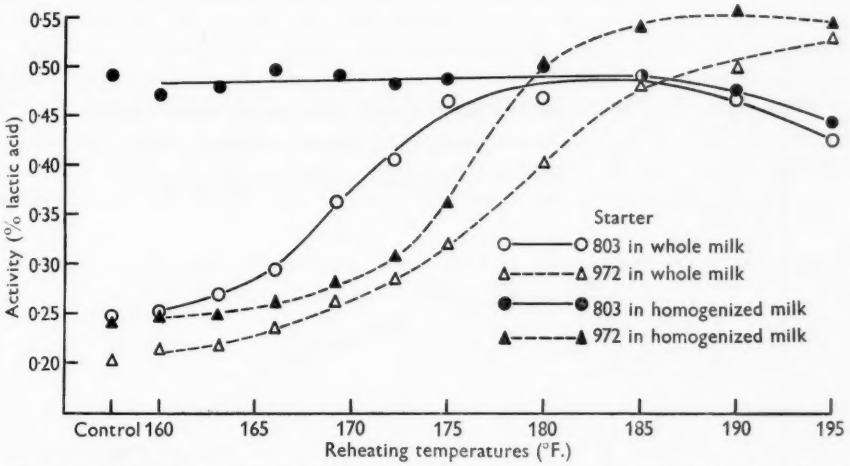

Fig. 1. Activity of starters 803 and 972 in H.T.S.T. and homogenized H.T.S.T. milk reheated to various temperatures.

that the factor causing inhibition of 972 has considerably greater heat resistance than the 'agglutinin', which is assumed to be responsible for the agglutination of organisms of the 803 type, and thus for the slowness when grown in pasteurized milk. In the work reported earlier (1) it was found that it was necessary to add more than 10 % of pasteurized milk to milk heated to high temperatures, or to homogenized milk in order to reduce the activity of the slow starters 760 or 803 in such milks. The addition of 5% of pasteurized milk was without effect, while an addition of 25% reduced the activities by about 50%. Since Auclair (5) had shown lactenin 1 and 2 to cause total inhibition in low concentrations, it was decided to assess the activity of 972 in mixtures of low- and high-temperature heated milks.

CHARACTERISTICS OF INHIBITION

Activity in mixtures of heat treated milks

Separated milk was heated to 185° F. in a small laboratory H.T.S.T. pasteurizer of 75 ml./min. capacity with a holding time of 20 sec., and aliquots of this heated milk were then steamed for 30 min. and others autoclaved at 5 lb./15 min. Increasing amounts of whole milk which had been heated in the same apparatus to 155° F. were added to the various separated milks and 10 ml. quantities filled into sterile test-tubes. These were cold-stored overnight and inoculated the following morning with starter 972. The activities obtained after 6 hr. storage at 30° C. are shown in Fig. 2.

Considering the total inhibition caused by the addition to autoclaved milk of 2–10% of milk heated to 155° F., the results are in good agreement with Auclair's findings. It is, however, remarkable that the addition of 155° F. milk to milk heated to 185° F., which is not inhibitory, should cause so much less inhibition, i.e. should not produce the characteristic sharp fall obtained with 1–2% additions to autoclaved or steamed milk. This point appeared of interest, and it was decided to see whether this difference would also be apparent in rennet whey prepared from such milks. Rennet wheys were, therefore, prepared from the whole milk heated to 155° F. and also from the separated milks heated to

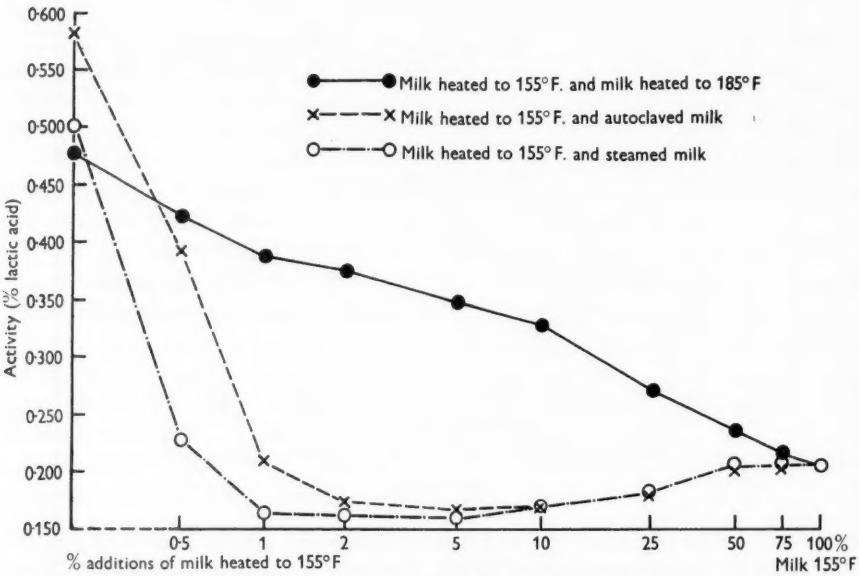

Fig. 2. Effect of various additions of milk heated to 155° F. to milks autoclaved, steamed or heated to 185° F. on the activity of starter 972.

185° F. and, autoclaved at 5 lb./15 min., respectively. Mixtures consisting of whey from milk heated to 155° F. and of whey from the two milks heated to high temperatures were inoculated with 972, and the pH values measured after 6 hr. at 30° C. with a Cambridge pH meter. The acidities were also determined, and the results obtained are shown in Table 1.

Table 1. *Activity of starter* 972 *in whey mixtures*

% rennet whey prepared from		Activity 6 hr. at 30° C.			
		pH (high-temperature wheys)		Acidity (high-temperature wheys)	
Whole milk 155° F.	Separated milk high temperature	185° F.	5 lb./15 min.	185° F.	5 lb./15 min.
100	Nil	5·29		0·160	
50	50	5·54	5·78	0·140	0·120
10	90	5·24	5·86	0·150	0·120
2	98	4·72	5·86	0·200	0·120
Nil	100	4·39	4·64	0·250	0·210
Uninoculated wheys		6·45	6·35	—	—

These results show that the inhibitory substance is present in the whey fraction, and that its effect is governed by the heat treatment applied to the milk from which the whey used as diluent has been prepared. The inhibitory action appears, therefore, to be dependent either on factors created in milk by steaming or autoclaving, or it may be assumed that reducing conditions created by heating milk to 190° F. may neutralize the inhibitory action. To clarify this, it was decided to oxidize this reducing system, which is measurable by the sodium nitroprusside test, by leaving the milk for a certain time in contact with small amounts of hydrogen peroxide and then removing any excess by catalase. The following experiment was set up.

Commercial homogenized H.T.S.T. milk was heated in a flask to 190° F. and cooled in water. Hydrogen peroxide was added to aliquots to give a final concentration of 100 and 30 p.p.m., respectively. These milks were first stored for 6 hr. at 22° C., after which time catalase was added, and the samples left overnight at about 15° C. Next morning, no residual hydrogen peroxide was detectable, and activity tests were carried out using starter 972 and fast starter 865 as control. The results are given in Table 2.

Table 2. *Effect on starter activity of oxidation by hydrogen peroxide of reducing compounds formed in milk by heating to* 190° F.

		Activity 6 hr. at 30° C.			
		Starter 972		Starter 865	
Milks used	Sodium nitroprusside	Milk only	Milk plus 2% H.T.S.T.	Milk only	Milk plus 2% H.T.S.T.
(1) Homogenized H.T.S.T. milk reheated to 190° F.	+ + +	0·590	0·610	0·620	0·660
(2) Milk '1' 100 p.p.m. H_2O_2 then catalase treated	0	0·315	0·180	0·305	0·305
(3) Milk '1' 30 p.p.m. H_2O_2 then catalase treated	0	0·310	0·190	0·305	0·325
(4) Commercial sterilized milk	—	0·610	0·240	0·580	0·630

These figures show that both starter activities are low in milks in which the reducing system, as measured by the sodium nitroprusside test, is oxidized by hydrogen peroxide. The inhibitory action when 2% of H.T.S.T. milk is added to such milks is very striking in the case of starter 972, but is not evident with 865. The characteristic marked difference in the effect of H.T.S.T. milk addition to sterilized and 190° F. heated milk is again demonstrated in milks 4 and 1. It would, therefore, seem likely that reducing compounds may be responsible for the curious lack of inhibition caused by the addition of H.T.S.T. milk to milk heated momentarily to about 190° F.

In view of the above findings, the activity of starters in ultra-high temperature milk (Uperized) together with the effect of H.T.S.T. milk additions was next examined. Controls consisted of commercial sterilized milk, and of homogenized H.T.S.T. milk reheated to 190° F. in a flask and cooled immediately. The results are shown in Table 3.

These figures show that Uperized milk supports starter growth well, but small additions of H.T.S.T. milk have neither such a striking inhibitory effect as with sterilized milk, nor such a negligible one as with milk heated momentarily to 190° F. Thus, Uperized milk which gives a positive turbidity test, a negative peroxidase test, and a positive nitroprusside test, behaves in an intermediate manner.

Table 3. *Activity of starter 972 in Uperized and other milks*

Milk	Homogenized H.T.S.T. milk additions (%)		
	Nil	0·6	2
Uperized	0·620	0·630	0·325
Sterilized	0·635	0·265	0·225
Homogenized 190° F.	0·650	0·690	0·650

Anaerobiosis

When milks were deaerated with nitrogen and a layer of liquid paraffin placed on the surface of the inoculated milk the inhibition was partially overcome. The effect in mixtures containing 50% and more of inhibitory milk was, however, negligible.

Acidification

Since Wilson & Rosenblum (8) mention that the inhibitory factor is damaged by the action of acids and alkalis, it was decided to investigate the action of acid on inhibitory whey. After a few preliminary experiments the following procedure was found satisfactory. Rennet whey was prepared from bulk raw milk and filtered through a Ford GS filter in order to obtain a clear and practically sterile source of inhibitor. 70 ml. quantities of this whey were acidified with varying amounts (0·6–1·0 ml.) of 3N hydrochloric acid and the pH measured. After 5 min. the whey was neutralized with 25% caustic soda and the pH again measured. At the same time the effect of this treatment on phosphatase and peroxidase was determined. Phosphatase was measured by the Aschaffenburg & Mullen test as modified by Tramer & Wight (9) and the peroxidase throughout this work was quantitatively determined by Tramer's modification (10) (DMS test) which is as follows.

Substrate: 0·25% in distilled water of dimethyl *p*-phenylene diamine sulphate (freshly prepared).

Buffer: 51·7 ml. of 0·1M potassium dihydrogen phosphate 48·3 ml. of 0·05M borax.

Hydrogen peroxide: 0·1%.

Precipitant: 10% acetic acid.

Procedure: to 10 ml. of buffer at room temperature add 2 ml. of milk and mix; then add 0·5 ml. of the substrate solution followed by 0·5 ml. of 0·1% hydrogen peroxide; invert and leave for exactly 1 min. Add 1 ml. of 10% acetic acid mix and allow to stand for 2 min. Filter through a Whatman No. 30 filter-paper and measure the colour of the

Table 4. *Effect of acidification on inhibitory effect and on phosphatase and peroxidase*

Sample	Rennet whey		Enzyme reaction of neutralized whey		Activity of 972 in sterilized milk containing neutralized whey	
	Acidified to pH	Neutralized to pH	Phosphatase A/M 2 hr.	Peroxidase *E* values	2%	10%
A	3·60	6·91	0·9	0·910	0·195	0·180
B	3·10	6·42	Nil	0·875	0·200	0·195
C	2·87	6·48	Nil	0·873	0·185	0·195
D	2·62	6·60	Nil	0·575	0·325	0·200
E	2·16	6·30	Nil	0·215	0·440	0·435
F	Untreated whey (control)		1·75	0·870	0·195	0·185
G	Sterilized whole milk		—	—	0·425	
H	Heated whey (185° F.)		—	0·076	—	

A/M = Aschaffenburg-Mullen units.

clear filtrate within 10 min. in a Spekker photoelectric absorptiometer (1 cm. cell) using green filter 604. Express results in *E* values.

These results show that phosphatase in whey is inactivated to a considerable degree by exposure to pH 3·60, and totally inactivated by exposure to pH 3·10 and below. Peroxidase is not affected by exposure to pH 2·82. Below this, however, inactivation starts, becoming very marked at pH 2·16, thus confirming other experiments which showed no detectable peroxidase after similar exposure to pH 1·4. The activities of 972 obtained when these neutralized wheys were added at two levels (2 and 10%) to commercial sterilized whole milk are shown in the last two columns. While the inhibitory effect of 2 and 10% additions is not influenced by acidification to pH 2·87, it is decreased as the pH falls until it is undetectable with whey acidified to pH 2·16. Although some peroxidase is still present in this whey, most of it had been inactivated by acidification. Thus, the experiments point to high correlation between inactivation of the inhibitory effect and of milk peroxidase by acidification. Further work was now directed towards establishing whether other factors causing the elimination of the inhibitory effect would also at the same time inactivate peroxidase.

Heat stability of inhibitor and of milk peroxidase

In view of the above findings and because peroxidase determinations had been carried out at that time, earlier results obtained with starter 972 in whole milk pasteurized in a 50 gal./hr. H.T.S.T. plant were re-examined, with the results shown in Table 5.

Table 5. *Relationship between peroxidase content of heated milks and the activity of* 972

Pasteurizing temperature (° F.)	DMS peroxidase test *E* values	Activity 6 hr. at 30° C.	Approx. residual peroxidase (%)
160·5	⩾1·000	0·225	100
165·5	>1·000	0·250	65
170·8	0·830	0·335	33
174·5	0·262	0·435	6·5
179·7	0·086	0·525	1
185·1	0·072	0·625	<1
203	0·065	0·610	<1

These results show that the activity of 972 increases with diminishing peroxidase values, suggesting high correlation between inactivation of the inhibitory action and of peroxidase by heat. The fact that good activity is obtained in the presence of as much as 6·5% residual peroxidase is presumed to be due to the SH compounds which are formed at these high temperatures of heat treatment.

Effect of hydrogen peroxide

Auclair (4) found that the lactenin titre of raw milk was reduced when milk was left in contact for some hours with hydrogen peroxide. The inhibitory power of lactenin was entirely lost when a concentration of 0·6% hydrogen peroxide was used. The author attributed this to the destruction of lactenin 2.

Peroxidase is known to be inactivated by hydrogen peroxide, and experiments were accordingly made to see whether the addition of hydrogen peroxide to sterilized milk containing a small percentage of inhibitory whey could made this milk less inhibitory.

1 ml. quantities of hydrogen peroxide of various concentrations (25–400 p.p.m.) were pipetted into sterile test-tubes, to which were then added 11 ml. of inoculated sterilized milk containing either 5 or 1% of rennet whey (source of inhibitor) or no whey (control). The activities were determined after 6 hr. at 30° C., and are shown in Fig. 3. The controls give the inhibitory action of hydrogen peroxide on the activity of 972 in sterilized milk. The acidity of 0·360 obtained in the presence of 1% inhibitory whey and 5 p.p.m. of hydrogen peroxide suggests a reaction between the inhibitory substance and hydrogen

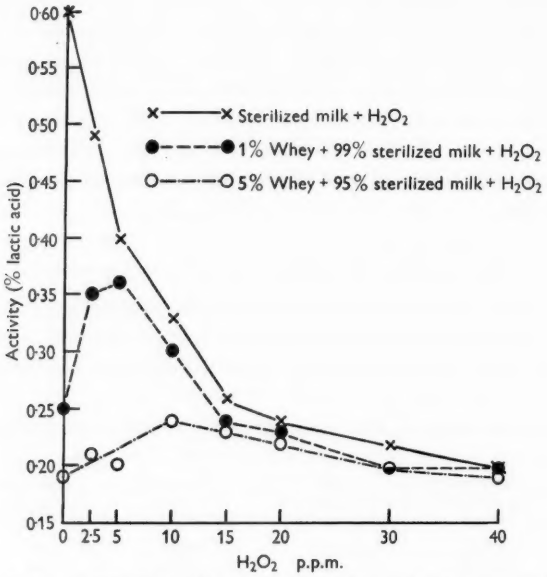

Fig. 3. Starter 972. Interaction between inhibitor and hydrogen peroxide.

peroxide. This is borne out by the experiment with 5% inhibitory whey present, where a small increase in activity was also obtained with 10 p.p.m. of hydrogen peroxide. However, the peroxidase test is not sufficiently sensitive to permit a study of peroxidase destruction at these low levels. A more satisfactory procedure was found in treating raw rennet whey (Ford GS filtered) with certain concentrations of hydrogen peroxide, and then adding catalase to destroy any excess.

In order to obtain a clear filtrate in the peroxidase determination, 1 ml. of whey to be tested was mixed with 1 ml. of sterilized milk and the usual D.M.S. procedure (10) applied. Catalase was determined by Anderson & Macwalter's modification (11) of the catalase test, with a reduced storage time. The catalase preparation used was prepared from beef liver (L. Light & Co. Ltd. Colnbrook, Bucks.) and contained 1,000 Kiel unit/g. The concentration of catalase found to be sufficient to decompose the residual hydrogen peroxide was about 0·001 Kiel units/ml. and this had no stimulatory action by itself. The inactivation of peroxidase in raw whey by the addition of hydrogen peroxide is a function of its concentration, contact time and storage temperature. Following preliminary experiments, a contact time of 30 min. with a storage temperature of 37° C. was chosen, and catalase

was added at the level of 0·001 Kiel unit/ml. The concentrations of hydrogen peroxide shown in Table 6 were so chosen as to cover intermediate points and 10% of the treated wheys were added to 90% of autoclaved milk.

Table 6. *Relationship between peroxidase content and activity of* 972 *in sterilized milk containing* 10% *hydrogen peroxide treated whey*

Rennet whey treated with H_2O_2 (final conc. p.p.m.)	DMS peroxidase test on treated whey *E* values	Activity in sterilized milk containing 10% treated whey
100	0·025	0·485
20	0·030	0·480
15	0·058	0·385
10	0·595	0·180
7·5	0·705	0·180
Nil (water)	—	0·185
Sterilized milk only	—	0·495

The activities suggest that the inhibitory factor is not affected by 10 p.p.m. of hydrogen peroxide, but 15 p.p.m. reduce its action very considerably, while 20 p.p.m. and more cause its complete elimination. The peroxidase inactivation appears to be affected in the same way, i.e. 10 p.p.m. having little effect whereas 20 p.p.m. appear to inactivate the enzyme completely. A high correlation is, therefore, shown to exist between the inactivation of the inhibitory factor by hydrogen peroxide and of peroxidase.

Effect of cysteine

Working with *Str. pyogenes*, Auclair & Hirsch (3) have shown that the inhibition due to lactenin can be entirely overcome by the addition of 10 mg. of cysteine per 100 ml. and partially by smaller amounts. In the experiments described below, 0·06–1·5 mg. of cysteine hydrochloride were added to 11 ml. of inoculated milk consisting of various quantities homogenized H.T.S.T. milk in sterilized milk. Fig. 4 shows that the addition of up to 0·3 mg. to sterilized milk had no effect itself, but the addition of 1·5 mg. caused some inhibition. In order to overcome the inhibition due to the 5% addition of homogenized H.T.S.T. milk, 0·3 mg./11 ml. of milk had to be incorporated; smaller amounts were found insufficient, while larger amounts (1·5 mg.) again caused inhibition. On the other hand, 50/50 mixtures of homogenized and sterilized milk require the addition of 1·5 mg. cysteine in order to overcome inhibition. The inhibitory action is thus counteracted by cysteine, but excess of either results in inhibition.

Effect of sodium hydrosulphite—$Na_2S_2O_4$

Sodium hydrosulphite was also found to overcome the inhibition caused by peroxidase-positive milk additions to autoclaved milk, but the action seems to be somewhat different from that of cysteine, although both are strong reducing compounds. A stock solution of sodium hydrosulphite was prepared and neutralized with NaOH before diluting as required. Various amounts of sodium hydrosulphite were incorporated in sterilized milk containing 2% of homogenized H.T.S.T. milk, and the activities carried out using starter 972. The results obtained showed that the addition of 1 ml. of M/500 or M/750 of sodium hydrosulphite to 10 ml. of milk gave values somewhat above that of the control; lower concentrations did not entirely overcome the inhibition and higher concentrations were inhibitory. In further work a final concentration of M/5000 was therefore chosen.

Having in mind the established relationship between the percentage of H.T.S.T. milk added, and cysteine (see Fig. 4), various mixtures of H.T.S.T. milk and sterilized milk were prepared and M/5000 sodium hydrosulphite incorporated. It was found that this concentration of sodium hydrosulphite overcame the inhibition caused by any quantity of homogenized H.T.S.T. milk. This is strikingly different from the action of cysteine, which is a well-balanced one, i.e. where a certain addition of H.T.S.T. milk requires a critical amount of cysteine for its 'neutralization'.

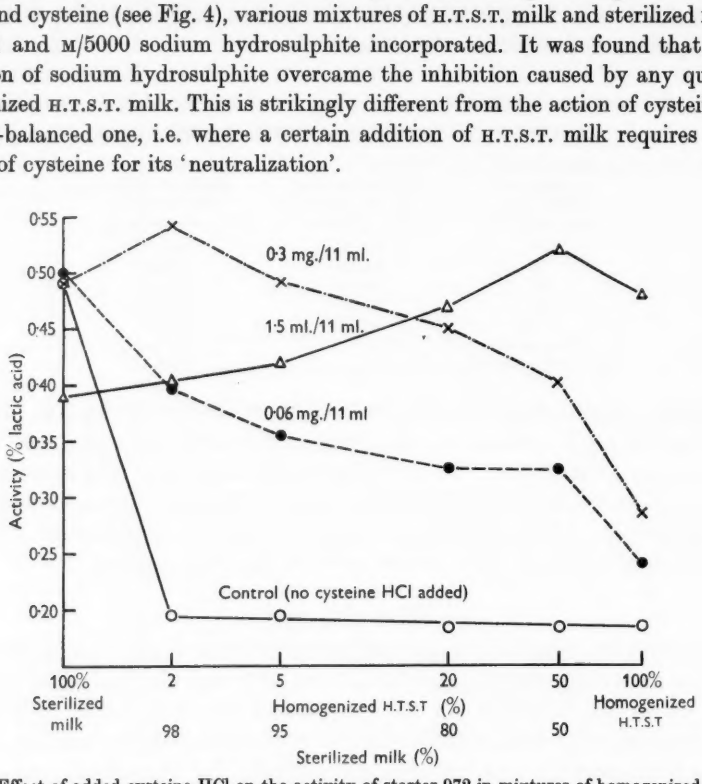

Fig. 4. Effect of added cysteine HCl on the activity of starter 972 in mixtures of homogenized H.T.S.T. and sterilized milk.

It was thought of interest to examine the influence of sodium hydrosulphite on other starters, especially on those which were, in our previous paper, shown to be carried up with the cream. The results obtained are shown in Table 7.

Table 7. *Influence of sodium hydrosulphite on the activity of various starters in different milks*

Starter	H.T.S.T. milk		Homogenized H.T.S.T. milk		Homogenized H.T.S.T. milk heated to 185° F.	
	Control	$+Na_2S_2O_4$	Control	$+Na_2S_2O_4$	Control	$+Na_2S_2O_4$
972	0·209	0·425	0·395	0·655	0·670	0·700
865 (fast)	0·725	0·735	0·720	0·715	0·690	0·680
803 (susceptible to creaming)	0·240	0·280	0·600	0·660	0·520	0·640
496 (susceptible to creaming)	0·280	0·310	0·685	0·680	0·565	0·640
738	0·370	0·510	0·360	0·575	0·545	0·585

Starters 972 and 738 are strongly activated by sodium hydrosulphite in homogenized H.T.S.T. and H.T.S.T. milk, but hardly at all in milk heated to 185° F. The fast starter 865 is unaffected and the two starters 803 and 496 are only very slightly stimulated by sodium

hydrosulphite in H.T.S.T. milk, suggesting that the depletion of the skim-milk of organisms is the predominant factor. It is unaffected by sodium hydrosulphite.

The relationship between acidities and cell numbers in homogenized H.T.S.T. milk with and without the addition of sodium hydrosulphite was next examined. The milk used was taken from the cooler exit of the heater and divided into two 100 ml. quantities. To one portion was added 2 ml. of Ringer solution to serve as control, while to the other was added 2 ml. of M/100 sodium hydrosulphite giving a final concentration of M/5000. Plate counts on tryptone, Yeastrel, lactose, beef extract agar and acidities were done at intervals. The results obtained are shown in Table 8. These figures show a close relation between acidities and cell counts, and suggest a partial bacteriostatic action in homogenized H.T.S.T. milk which is overcome by sodium hydrosulphite.

Table 8. *The influence of sodium hydrosulphite on the growth of* 972 *in homogenized milk*

	Acidity		Plate counts $\times 10^6$	
After hr. at 30° C.	Control	$Na_2S_2O_4$ added	Control	$Na_2S_2O_4$ added
0	0·145		5·6	
2	0·145	0·145	14·4	21·9
3	0·160	0·195	—	—
4	0·170	0·265	24·6	134·0
5	0·205	0·400	—	—
6	0·260	0·680	91·0	227·0

It is of interest to compare these figures with those obtained with commercial sterilized milk with and without an addition of 2% H.T.S.T. milk (Table 9).

Table 9. *Acidities produced by starter* 972 *in sterilized milk with and without an addition of* 2% *high temperature and short time milk and corresponding plate counts*

	Experiment 1				Experiment 2			
	Sterilized milk		Sterilized milk with 2% H.T.S.T. milk		Sterilized milk		Sterilized milk with 2% H.T.S.T. milk	
After hr. at 30° C.	Acidity	Plate count ($\times 10^6$)	Acidity	Plate count ($\times 10^6$)	Acidity	Plate count ($\times 10^6$)	Acidity	Plate count ($\times 10^6$)
0	0·140	4·5	0·140	4·5	0·145	6·0	0·140	6·0
2	0·155	12·4	0·140	4·9	0·160	20·5	0·160	6·6
4	0·220	47·0	0·155	4·8	0·255	56·0	0·165	5·7
6	0·425	119·0	0·155	2·7	0·485	162·0	0·165	2·7

The results in Tables 8 and 9 agree with those of Auclair, who found that *Str. agalactiae* is very sensitive to lactenin when raw milk is diluted in sterilized milk, but not in the same undiluted raw milk. He suggested that this difference was due to the presence of stimulatory substances in the raw milk.

Effect of sodium azide

It is well known that sodium azide inactivates catalase, that it is inhibitory to non-pathogenic streptococci and certain other organisms, and that the critical concentration is 1:15,000 (approx. M/1000). In view of the possible similarity in structure of catalase and peroxidase the effect of azide on the latter was investigated.

Preliminary experiments using 972 showed that an azide concentration of M/2000 is inhibitory, that M/20,000 has hardly any effect, while M/200 results in total inhibition. Next it was found that azide inhibits milk peroxidase instantaneously and that the inhibition is a function of the concentration. Having these preliminary findings in mind, the following experiment was set up.

10% additions of various strengths of sodium azide (M/100–M/3200) were made to homogenized H.T.S.T. milks, and their peroxidase contents were then determined. Sterilized milks treated similarly served as controls. 5% of these treated milks, i.e. both homogenized and sterilized, were added to 95% of pure sterilized milk, and the usual activities carried out using starter 972. The results are shown in Table 10.

Table 10. *The relationship between peroxidase content and the activity of* 972 *in sterilized milk containing* 5% *of azide treated milk*

Sodium azide concentration in homogenized H.T.S.T. milk	Residual peroxidase after azide treatment		Activity in 5% azide treated H.T.S.T. in autoclaved milk	Calculated amount of peroxidase in test mixture (approx. %)	Activity in control (no H.T.S.T. milk present)
	E values	(Approx. %)			
M/1000	0·183	8	0·445	0·4	0·445
M/2000	0·194	8·2	0·460	0·41	0·465
M/4000	0·202	8·5	0·365	0·425	0·455
M/8000	0·300	14	0·275	0·7	0·460
M/16000	0·445	20	0·220	1	0·460
M/32000	0·715	>20	0·220	>1	0·450
Water	1·4	100	0·220	5	0·470

The results in Table 10 show a close inverse correlation between activity and residual peroxidase. The presence of about 1% undestroyed peroxidase caused total inhibition, 0·4% had no appreciable effect and values in between gave intermediate activities. The controls show that the presence of M/20,000 azide has no effect on the activity. It is of interest that this sulphur free enzyme poison is capable of inactivating both the inhibitor and milk peroxidase to the same degree.

Comparison between a lactenin-susceptible starter and starter 972

In view of the close similarities between the results of Auclair *et al* using a particular starter, *Str. lactis* M_1, and many of the results reported in this paper, it was felt of interest to examine M_1 further. A culture of this strain was kindly supplied by the N.I.R.D. Shinfield, and the results obtained in various milks with this starter, with starter 972, and also with 865 (fast control), are given in Table 11. The trend in activity of starters M_1 and 972 in the various milks used appears very similar, the main difference being that 972 is much more strongly affected by the inhibitor in question than M_1. The control starter 865 is unaffected.

The influence of colostrum

Auclair & Hirsch (3) examined the inhibitory effect of colostrum and reported that it was rich in lactenin 1 and poor in lactenin 2. Because of the close relationship between some of the work reported in this paper and that of these authors, colostrum has been examined.

It was clearly of importance to establish the peroxidase content of colostrum in relation to that of normal milk. Preliminary tests showed that colostrum taken on the day of

Table 11. *Comparison of the activity of M_1 and two other starters in various milks*

Milks	Activity of starters		
	M_1	972	865 (fast)
(1) H.T.S.T.	0·450	0·250	0·710
(2) Homogenized H.T.S.T.	0·570	0·350	0·740
(3) Commercial sterilized	0·620	0·640	0·645
(4) H.T.S.T. reheated to 190° F. (momentarily)	0·610	0·700	0·700
(5) Homogenized H.T.S.T. reheated to 190° F. momentarily	0·620	0·645	0·700
(6) 2% H.T.S.T. + 98% milk 4	0·630	0·700	0·690
(7) 2% H.T.S.T. + 98% milk 5	0·640	0·635	0·685
(8) 2% H.T.S.T. + 98% milk 3	0·330	0·190	0·670

calving (24) contained only about one-fifth of that of normal milk, whereas colostrum taken from the same cow 4 days later (28) had risen to the average level.

The effect of the presence of colostrum on the activity of 972 in sterilized milk was examined, and actual peroxidase values established. These results are given in Table 12.

Table 12. *Peroxidase content of and the activity of* 972 *in sterilized milk containing varying amounts of colostrum*

% colostrum	Colostrum 24		Colostrum 28	
	Activity	Peroxidase (*E* values)	Activity	Peroxidase (*E* values)
0	0·505	0·033	0·505	0·033
0·5	0·495	—	0·390	—
1	0·470	—	0·260	—
2	0·455	0·034	0·255	0·066
5	0·280	0·043	0·235	0·207
10	0·330	0·067	0·235	0·488

These figures show again a high correlation between the amount of peroxidase and the degree of inhibition.

Opportunity was taken to study the so-called lactenin 1 content of colostrum taken on the day of calving. Such colostrum always possesses an abnormally high globulin content and thus would be rich in 'agglutinin'. This was confirmed by experiments in which various amounts of colostrum 24 were added to whole milk which had been heated momentarily to 80° C. in order to destroy its creaming property. The results obtained with two starters (760 and 803), previously found to be very susceptible to agglutination, are given in Table 13.

Table 13. *Effect of various additions of colostrum* 24 *to milk heated to* 80° C *on its creaming property and activity of starters* 760 *and* 803

Milk 80° C. plus colostrum 24 (%)	Cream line % (at room temperature) (hr.)					Activity 6 hr. at 30° C. (starters)	
	½	1	2	4	6	803	760
0	0	½	¾	¾	1	0·480	0·520
0·3	0	Tr	½	¾	1¼	0·395	0·455
0·6	0	Tr	1	2¼	3½	0·305	0·375
1·0	0	½	3½	5½	5¾	0·270	0·325
2·0	½	5½	8¼	8½	8¾	0·240	0·285
Milk heated to 63° C./25 min.	½	4¼	5¾	6	6½	0·235	0·250

Earlier in this paper it was stated that it was necessary to add 10% H.T.S.T. pasteurized milk to milk which had been heated sufficiently to destroy its creaming properties, in order to effect any reduction in the activity of such starters as 760 and 803, i.e. those affected by cream rising. Table 13 shows that colostrum, taken on the day of calving, is a very potent source of 'agglutinin', containing probably ten times as much as normal milk.

DISCUSSION

The work reported in this paper strongly suggests that slowness in certain starters is associated with milk peroxidase activity. Support for this view is found in published work on the bactericidal properties of milk. Hanssen (12) may have been the first to appreciate this for he states that 'the bactericidal properties of milk are in close relation to the oxidizing enzymes of the milk'. He based this observation on the relationship between the heat treatment of the milk as indicated by the original Storch peroxidase test and its bactericidal properties. Jago (6) also suggested that inhibition might be enzymic in nature.

In considering the relationship between peroxidase and slowness in certain cheese starters, it seems likely that the inhibition is due to an oxidation product formed as the result of peroxidase activity. In turn this product can only be formed in the presence of a peroxide. It is well known that certain streptococci can produce hydrogen peroxide, and it has been suggested that this has an inhibitory effect only if the particular strain concerned is catalase negative. We have examined various fast and slow starters, but no real difference has been noted in their catalase content or in the effect upon them of traces of hydrogen peroxide.

The formation of an inhibitory oxidation product by milk peroxidase in the presence of hydrogen peroxide is in keeping with the fact that peroxidase action in the presence of peroxides results in the formation of compounds having a quinonoid structure (Saunders & Watson (13)). Such substances can be very inhibitory when present in milk and it is assumed that they must exhibit a considerable degree of specificity, in order to explain the difference between fast and certain slow starters. Alternatively, it is possible that fast starters do not produce traces of hydrogen peroxide, but this cannot be due to the action of catalase, for the breakdown of hydrogen peroxide by catalase is very much slower than that due to peroxidase. It is interesting to note that the normal milk peroxidase test can readily be reversed by reducing compounds, i.e. the colour produced disappears when certain reducing compounds are added, e.g. cysteine, sodium hydrosulphite, but the time necessary for the decolorization varies with the substrate and the reducing compound used. Since Saunders has shown that the coloured compound produced in the peroxidase test is a quinone, it is reasonable to assume that the action of reducing compounds, such as cysteine and sodium hydrosulphite, leads to an instantaneous reduction of the inhibitory oxidation product.

It is now pertinent to consider why no inhibition occurs with this type of starter in the presence of milk which has been heated to 185–190° F. and to which has been added 1–2% of pasteurized milk, whereas it does occur in sterilized milk containing 1–2% of pasteurized milk. The work reported here seems to show that inhibition is not found because of the presence of SH compounds developed during heat treatment to 190° F. If these compounds—as measured by the nitroprusside test—are oxidized by hydrogen peroxide, the milk behaves like sterilized milk, i.e. the inhibition occurs. The action of

added cysteine and sodium hydrosulphite is thus similar to that of SH compounds formed by heat treatment. It should be noted, therefore, that whilst the inhibition can be overcome by sodium azide, this acts in a different way, for peroxidase is inactivated by azide, and thus no inhibitory oxidation product can be formed.

Auclair attributes the inhibitory properties of milk towards certain organisms to two substances, lactenin 1 and 2, the word lactenin having been first used by Jones (14) to describe the substance responsible for the inhibitory property of milk towards streptococci and in particular *Str. pyogenes*. In a previous paper (1) the apparent inhibitory effect of pasteurized milk on the growth of certain cheese starters has been shown to be due to an 'agglutinin', and it is now suggested that lactenin 1 and this agglutinin are identical. Furthermore, from the work presented it would appear that the inhibition ascribed to lactenin 2 is the same as that brought about by peroxidase action. This is borne out by published work and is confirmed by our results obtained with starters M_1 and 972. It is thought that the removal of this type of inhibition by various means may fall under two headings, i.e. inactivation of peroxidase and reduction of the postulated inhibitory oxidation product. While heat, lowering of pH, hydrogen peroxide and azide will inactivate the enzyme, cysteine or sodium hydrosulphite can be assumed to reduce the oxidation product. Finally, it is interesting to note that many of those who have studied this problem of slow cheese starters have stated that the inhibitory effect disappears if milk is heated to 71° C. for 30 min. Our findings show that such treatment is just sufficient to inactivate peroxidase in milk.

SUMMARY

1. Certain starter cultures have been found to be inhibited in the presence of milk peroxidase.

2. A high correlation has been established between the inactivation of milk peroxidase and of the inhibitory action by use of critical values of any of the following: temperature, pH, concentration of either sodium azide or hydrogen peroxide.

3. The inhibitory action can be overcome by the addition of certain reducing substances such as cysteine and sodium hydrosulphite.

4. The inhibitory action is exhibited by the corresponding separated milk and whey.

5. It is suggested that this inhibition may be due to the formation of a specific inhibitory oxidation product having a quinonoid structure.

6. The addition of 1–2% of pasteurized milk or whey to autoclaved milk caused total inhibition, whereas such additions to milk heated to 190° F. showed no such effect. This is attributed to the presence of SH groups in the latter milk capable of reducing the oxidation product.

7. It is suggested that the inhibition due to peroxidase is identical with that ascribed to lactenin 2, and that the inhibition described in our previous paper as due to 'agglutinin' is identical with that ascribed to lactenin 1.

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701. A COMPARISON OF DRIED SKIM-MILK AND WHITE FISH-MEAL AS PROTEIN SUPPLEMENTS FOR FATTENING PIGS

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The high value of skim-milk, both in liquid and dried forms, as a protein supplement for pigs is well established. Its use in practice, however, as a replacement for other protein supplements such as white fish-meal must depend both on the relative value of the protein supplied and on the market price of the supplements.

The object of the experiment described here was to obtain further data on the comparative value of dried skim-milk and white fish-meal as protein supplements in feeding trials with fattening pigs.

EXPERIMENTAL

(1) *Treatments*

There were four treatments (see Table 1).

(i) Meal containing 10% white fish-meal, reduced to 7% from 130 lb. live weight.

(ii) Fish-meal in (i) replaced by 10 and 7% dried skim-milk respectively (i.e. weight for weight basis).

(iii) Fish-meal in (i) replaced by 15 and 10½% dried skim-milk, respectively, with appropriate adjustments in the barley-meal percentages.

(iv) Fish-meal in (i) replaced by 20 and 14% of dried skim-milk, respectively, with appropriate adjustments in the barley-meal percentages.

(2) *Diets*

The composition of the meal mixtures used is given in Table 1, together with their calculated crude protein* and starch equivalent values. In Table 2, the percentage crude protein, calcium, phosphorus and salt in white fish-meal and dried skim-milk are shown. It is seen from Table 1 that the total crude protein content was approximately the same for the diets used on treatments 1 and 4, whereas in the diets given to the pigs on treatments 2 and 3, the total crude protein was lower corresponding to the amount of skim-milk included in them. In order that the total calcium and phosphorus content of the diets containing skim-milk should be approximately the same as that of the fish-meal diets, steamed bone flour was added to them.

(3) *Pigs*

Thirty-six Large White weaners, 9–11 weeks old at the start of the experiment, were selected from seven litters, from the virus-pneumonia free herd at Shinfield, and were divided into nine blocks of four litter-mate pigs each. The pigs were as far as possible of

* Using the figures in Table 2 for the white fish-meal and dried skim-milk, and figures given by Woodman (1) for the other ingredients.

Table 1. *Percentage composition of meals used on the four treatments, together with calculated crude protein and starch equivalent values*

Treatment ...	1		2		3		4	
	To 130 lb. live weight	130 lb.—slaughter	To 130 lb. live weight	130 lb.—slaughter	To 130 lb. live weight	130 lb.—slaughter	To 130 lb. live weight	130 lb.—slaughter
Fine miller's offal	38	28	38	28	38	28	38	28
Barley meal	52	65	50½	64	45¾	60½	41	57½
White fish-meal	10	7	—	—	—	—	—	—
Dried skim-milk	—	—	10	7	15	10½	20	14
Steamed bone flour	—	—	1½	1	1¼	1	1	¾
Aurofac 2A*	2½ lb./2000 lb. in all meals							
Rovimix†	4½ g./100 lb. to 130 lb. live weight; 2 g./100 lb. from 130 lb. live weight to slaughter							
% crude protein	17·7	15·5	14·9	13·5	16·3	14·5	17·7	15·5
Starch equivalent	63·9	65·9	65·3	66·9	66·1	67·3	66·8	67·9
Starch equivalent ratio / Crude protein	3·61	4·25	4·38	4·96	4·06	4·64	3·77	4·38

* Cyanamid of Great Britain Ltd., containing 3·6 g. chlortetracycline/lb.
† Roche Products Ltd. containing 50,000 i.u. vitamin A and 12,500 i.u. vitamin D_3/g.

similar initial weight and there were five male and four female pigs on each treatment, except treatment 4 on which there were four males and five females.

The four experimental treatments were allocated at random to the four pigs in each of the blocks, each block of pigs being housed in one pen in a Danish-type piggery and individually fed. The first block of four pigs started on 13 September 1955 and the last block on 25 October 1955. All pigs were wormed shortly after weaning and were given approximately 1 week to settle down in the individual feeding pens and to change over to the experimental diets before the experiment proper began.

Table 2. *Percentage crude protein, calcium, phosphorus and salt in white fish-meal and dried skim-milk*

	White fish-meal	Dried skim-milk
Crude protein	66·1	38·4
Calcium	6·04	1·30
Phosphorus	3·41	1·04
Sodium chloride	1·62	1·86

The pigs were weighed weekly and were fed according to live weight and a scale described by Braude & Mitchell (2) up to a maximum of 6½ lb./pig/day; the adjustments in the meal allowances were made once weekly after weighing. The meal was given as a wet mash, 3 lb. of water being added to each 1 lb. of meal just before feeding.

The pigs were sent to slaughter during the week when, on the normal weighing day, they reached a live weight of 204 lb. or above.

In addition to the normal factory grading, the following measurements were made on the right-hand side of all carcasses on the day of slaughter: length, back-fat thickness at shoulder, mid-back and loin, and belly thickness. After curing and smoking, eye muscle breadth and depth on the cut side was determined. All measurements were made according to the method described by McMeekan (3), his loin and rump fat measurements corresponding to our mid-back and loin fat measurements, respectively.

RESULTS

(1) *Growth and food utilization*

The results are given in Table 3. All the pigs on experiment were very thrifty, growing at a consistent rate and remaining in excellent health throughout the experiment. The data were submitted to simple analyses of variance, it having been shown previously (Barber, Braude & Mitchell (4)) that the method adopted in arranging the blocks of pigs gave

Table 3. *Mean live-weight gain and efficiency of food utilization of pigs during fattening period beginning at 9–11 weeks of age*

Treatment no.	...	1	2	3	4
No. of pigs	...	9	9	9	9
Initial weight (lb.)		46·7	46·8	46·3	47·3
Final weight (lb.)		209·1	209·4	207·2	208·9
Days on experiment		111·2	115·1	109·7	105·0
Daily gain (lb.)*		1·47	1·42	1·47	1·54
Food consumed (lb./pig)		512·6	520·5	497·3	482·6
Food consumed (lb./lb. live weight gain)*		3·16	3·20	3·09	2·99

* The differences between means necessary for statistical significance were:

	Level of significance			Standard error	Coefficient of variation (%)
	5%	1%	0·1%		
For daily gain	0·073	0·099	0·132	±0·025	5·09
For amount of food consumed per lb.	0·150	0·204	0·273	±0·052	4·97

satisfactory control of the unavoidable differences in initial weight and that adjustment by covariance was unnecessary. The figures in Table 3 show that the only significant difference in growth rate was between the pigs on treatment 4 receiving 20% dried skim-milk and those on treatment 2 given the diet with 10% dried skim-milk (treatment 4 > 2 at 1% level of significance). The difference between treatment 4 and treatments 1 and 3 approached significance at the 5% level.

These differences in growth rate were reflected in the efficiency of food utilization results, the pigs on treatment 4 (20% skim-milk) converting their food significantly more efficiently than the pigs given the 10% dried skim-milk diet on treatment 2 (1% level of significance), and those on treatment 1 receiving the fish-meal diet (5% level of significance). None of the other treatment differences reached the 5% level of significance.

(2) *Carcass measurements*

The results are given in Table 4. Again the data were submitted to simple analyses of variance, Barber *et al.* (4) having previously shown that the system of sending the pigs to slaughter gave sufficient control of the influence of dead weight on the various carcass measurements so that adjustment by covariance was unnecessary.

Of all the carcass measurements made, only in the case of shoulder fat and belly 3 did treatment differences reach the 5% level of significance.

The pigs on treatment 1 had a significantly thinner shoulder fat than those on both treatments 2 and 3 (5% level of significance) and a significantly thinner belly 3 measurement than the pigs on treatment 3 (1% level of significance).

Table 4. *Mean carcass measurements and commercial grading‡ results*

Treatment no. ...	1	2	3	4
No. of pigs ...	9	9	9	9
Cold carcass weight (lb.)	155·6	156·8	153·8	155·0
Carcass length (mm.)*	779·3	774·2	771·0	783·9
Shoulder fat (mm.)*	46·9	50·6	51·1	48·7
Mid-back fat (mm.)*	24·6	25·8	25·4	23·1
Loin fat (mm.) 1*	33·8	32·9	33·0	32·2
2*	26·6	28·7	28·8	27·0
3*	35·6	38·6	38·0	37·0
Belly thickness (mm.) 1*	31·0	34·4	33·6	32·9
2*	26·9	30·8	30·1	28·0
3*	28·4	30·9	33·7	31·0
Eye muscle breadth (mm.)*	87·7	85·4	84·7	83·3
Eye muscle depth (mm.)*	38·3	39·0	38·4	37·4
Commercial grading:				
No. of final grade A carcasses	9	6	9	8
No. of final grade B carcasses	0	3	0	1
No. of triple A carcasses	7	5	6	5

‡ As in force when the pigs were slaughtered.

* The differences between means necessary for statistical significance were:

	Level of significance			Standard error	Coefficient of variation (%)
	5%	1%	0·1%		
For carcass length†	13·70	—	—	±4·69	1·81
For shoulder fat	3·21	4·35	—	±1·10	6·70
For mid-back fat†	2·92	—	—	±1·00	12·15
For loin fat 1†	3·04	—	—	±1·04	9·47
2†	2·79	—	—	±0·96	10·35
3†	3·16	—	—	±1·08	8·72
For belly thickness 1†	3·83	—	—	±1·31	11·94
2†	4·79	—	—	±1·64	17·01
3	3·50	4·74	6·34	±1·20	11·60
For eye muscle breadth†	4·53	—	—	±1·55	5·46
For eye muscle depth†	3·75	—	—	±1·29	10·08

† Mean square not significant at 5% level.

Commercial grading

The final grading of the individual pigs on all four treatments was very good, with no marked differences between any of the treatments (see Table 4).

DISCUSSION

The results obtained in this experiment show that there was no significant difference in rate of growth or efficiency of food conversion when a supplement of 10 reduced to 7% of fish-meal, was replaced on weight per weight basis by dried skim-milk (comparison of treatments 1 and 2). On the other hand, when the fish-meal supplement was replaced by dried skim-milk on protein basis, the pigs receiving the dried skim-milk grew faster (approaching 5% level of significance) and utilized their feed significantly more efficiently (comparison of treatments 1 and 4). When one takes treatment 3 also into consideration, the mean figures suggest a trend towards slightly improved growth rate and efficiency of food utilization with increasing levels of dried skim-milk in the diet (differences between treatments 4 and 2 being statistically significant).

These results suggest that the protein in the dried skim-milk is of greater value to the pig than that in fish-meal. There are, however, at least two points which must be considered before accepting this interpretation.

In the first place, there is good evidence that the supplies of protein in a diet, such as that used here containing 10 % white fish-meal, are very generous. Woodman & Evans (5), for example, showed that pigs given a diet containing only 7 % fish-meal up to 90 lb. live weight and none thereafter, did as well as those receiving a diet with 10 % fish-meal up to 150 lb. live weight. It is likely, therefore, that the pigs on treatment 1 in this experiment received more protein than they required.

Secondly, the higher energy value of dried skim-milk compared with white fish-meal should be taken into consideration. As seen from Table 1, all the skim-milk diets were higher in starch equivalent than the corresponding fish-meal diets, so that other things being equal some improvement in performance is to be expected from this fact alone. The trend for improved performance with increasing skim-milk in the ration shown in Table 3 is undoubtedly a reflexion, at least in part, of these differences in energy value of the various rations.

It may well be, however, that the diet with only 10 % dried skim-milk supplement is rather marginal from the point of view of covering the protein requirements of young fattening pigs, and that better results may, in general, be obtained from a 15 % supplementation. In this connexion, one should take into consideration the fact that the total protein in the ration is composed not only of protein contributed by the skim-milk, but also of the protein contributed by the cereals. The latter may vary very considerably, and sometimes the augmentation by 10 % skim-milk may be sufficient to cover the total protein requirements of the pig; on other occasions, however, it may fall short of it and in such cases the inclusion of the higher percentage of dried skim-milk should prove worth while.

In addition to the significantly thicker shoulder fat of the pigs on treatments 2 and 3 compared with those on treatment 1, the mean figures in Table 4 show a trend in the case of most of the other back-fat measurements for the skim-milk fed pigs, particularly those on treatments 2 and 3, to have slightly thicker fat measurements than the animals given the fish-meal diets.

It might be that these differences are a reflexion of the higher energy/protein ratios in the skim-milk as compared with the fish-meal diets, (see Table 1) and that while in the pigs used in this experiment such differences were not reflected in the commercial grading results, in pigs with a lower grading potential, some adverse effects on carcass quality might arise.

The tendency for thicker bellies in the pigs fed the skim-milk compared with the fish-meal diets, might similarly be a reflexion of the differences in the energy/protein ratios. However, since, as with the back-fat measurements, statistically significant differences at the accepted level occurred in only one case, clearly further work is required on these questions before any final conclusions can be drawn.

SUMMARY

1. Thirty-six 9–11-week-old Large White pigs in nine blocks of four litter-mates were individually fed on diets containing either white fish-meal or various amounts of dried skim-milk as the protein supplement. The pigs were fed according to live weight and a scale up to a maximum of 6½ lb. of meal/pig/day.

2. There were no significant differences in rate of growth or efficiency of food utilization between pigs given the diets containing as protein supplements either 10 % white

fish-meal reduced to 7% at 130 lb. live weight, 10% dried skim-milk reduced to 7%, or 15% dried skim-milk reduced to 10½%.

There was, however, a trend towards an improvement in both rate of growth and efficiency of food utilization with increasing levels of skim-milk in the ration, and the differences between the high skim-milk diet (20 reduced to 14%) and the low (10 reduced to 7%), and also between the former and the fish-meal diet, reached statistical significance.

3. The difficulties of drawing any definite conclusions from the results about the relative value of the protein in white fish-meal and dried skim-milk were discussed in relation to (*a*) the probability that the white fish-meal diet used contained over-generous supplies of protein, and (*b*) the higher energy value of dried skim-milk as compared with white fish-meal.

4. The commercial grading of pigs on all four treatments was very good. The carcass measurement data indicated a tendency for the skim-milk diets, particularly those containing either 10 or 15%, to result in some increase in both back fat and belly thickness. The possibility that this was associated with the higher energy/protein ratios in the skim-milk as compared with the white fish-meal diets was discussed.

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REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION C. DAIRY CHEMISTRY

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PART I. GENERAL AND PHYSICAL CHEMISTRY

As milk proteins and enzymes formed the specific subjects of the previous Review(1), other topics covered in earlier Reviews have not been discussed for some 4 years. Some of these topics are, therefore, considered in the present contribution using papers which appeared, for the most part, between 1953 and the early months of 1957.

In preparing the Review, the author has been struck by the fact that, of recent years, the quality of publications has shown an inverse trend to their ever-increasing quantity. The six heavy tomes incorporating the proceedings of the XIVth International Dairy Congress are quoted as an illustration. They speak volumes! Greater self-restraint and self-criticism on the part of authors, and greater discrimination on the part of editors are the obvious remedies.

(1) PHYSICO-CHEMICAL PROPERTIES OF MILK

Some of these vary so widely under normal conditions, and are enhanced or diminished by so many factors during treatment and processing that little profit can be derived from their measurement. The electrical conductivity of milk provides a good example. As shown in an excellent review by Schulz(2) who summarizes the literature from its initiation in 1891, data are plentiful, but so discouraging that new attempts to gain information by conductivity testing have almost ceased. Montefredine & Laporta(3) have, on the basis of old data, drawn attention to the marked inverse relationship between the conductivity and specific weight of milk.

(*a*) pH. Here too, fresh reports are scarce. Results for Indian cows and buffaloes have been recorded(4) as well as lactational trends and changes due to heat treatment(5). Buffalo milk was more strongly buffered in the acid range, and more weakly buffered in the alkaline range than cows' milk(6).

(*b*) *Ionic calcium and magnesium.* Elucidation of the equilibria governing the distribution of milk calcium and magnesium, present in the ionic form and in association with inorganic and organic constituents, provides a challenging subject for study, and it is gratifying to report some

progress in this complicated field. Two teams, Christianson, Jenness & Coulter in America (7) and van Kreveld & van Minnen in Holland (8) have, by techniques differing in detail but based on the same principle, determined the ionic concentration of calcium and magnesium in milk. Using different cationic exchange resins, and working under conditions designed to disturb the natural equilibrium as little as possible, they arrived at almost identical ionic concentrations (in m-mole/l.)

Reference	Ca	Mg
(7)	2·0–2·3	0·82–0·85
(8)	2·2 (mean)	0·8 (mean)

Corresponding values calculated from the results of analyses of milk ultra-filtrates are generally somewhat higher. Thus, mean values for calcium of 2·75 and 2·9 m-mole/l. have been reported by Smeets (9) and White & Davies (10), respectively. Taken together, these findings provide strong evidence that only a small proportion of milk calcium and magnesium is present in the ionic form, and this proportion is reduced even further by heat treatment (7, 8), by increases in pH or by the addition of citrate (7) or phosphate (8). Valuable as these studies are, they also serve to emphasize the limitations of work that can only provide information about a small proportion of the total calcium and magnesium of milk.

An entirely different approach was chosen by Gehrke and his colleagues who have studied the rate of removal of calcium from skim-milk by an excess of ion exchange resin (11). More than 90% of the total calcium was exchanged after contact for 180 sec. The authors deduce that milk contains about 6 m-mole/l. of calcium in the ionic form, a much higher concentration than that obtained with ion exchange resins under equilibrium conditions. However, a similarly high value is calculated elsewhere (12) from the results of experiments with membrane electrodes whose construction and testing are described in a separate paper (13). Technological aspects of the effects of ion exchangers on milk have been considered by Murthy & Whitney((14) who have studied the removal of various ions by cation and anion exchange resins separately and in combination, and by Coleman, Bishov & Mitchell (15) who concerned themselves more specifically with the changes in pH and flavour resulting from the removal of calcium. Bishov & Mitchell (16) made further use of ion exchangers in a study of changes in the sedimentation behaviour of milk proteins resulting from alterations in the mineral balance. The possibilities of mitigating the effects of milk souring by treatment with anionic exchange resins have been discussed by Italian workers (17).

(*c*) *Density*. Full descriptions of versions of the displacement technique suitable for high precision work on the density of milk have been given by Short (18) and by Rutz, Whitnah & Bactz (19). Taking precautions to ensure uniformity in the physical state of the fat (though those adopted by Rutz *et al.* (19) have been the subject of critical comment by Short (20)), their results show, in agreement, that the density of milk falls slowly as the temperature is raised from 10 and 0° C., respectively, to 45° C. The results of Short are wider in scope, dealing with milks varying in fat content from 0·02 to 6%, whilst the American workers used milk standardized to 4% fat. Rutz *et al.* (19) observed no significant changes in density when milk was homogenized under pressures varying from 500 to 3000 lb./sq.in., whereas Short (21) found small, but significant, increases after processing at 3000 lb./sq.in. Pasteurization does not affect the density of raw milk, according to Short, whilst sterilization of homogenized milk leads to some reduction in the density values. Making use of super-cooling, the American workers later extended their work to temperatures below 0° C. (22), and observed maximum densities at about −5·5° C. with homogenized milk, and at about −2·75° C. with skim-milk. Yugoslav workers have summarized data obtained by determining the specific gravity of 2670 herd milks (23), and of milks from twenty single cows for which a total of over 20,000 readings was recorded (24).

(*d*) *Viscosity*. Whitnah, Rutz & Fryer (25) have measured the viscosity of pasteurized, homogenized milks, and the changes in such milks stored at 4° C. Viscosity was found to increase linearly with the logarithm of the time of storage. The increases were much more pronounced in fresh skim-milk than in reconstituted skim-milk powders, some of which did not alter in viscosity on storage (26). A linear relation between viscosity and total solids is suggested by some data of

Goded Y Mur(27) who reconstituted skim-milk powder to fluids of varying solids content. Kulkarni & Dole(28) provide data on the viscosity of the milk of the buffalo, goat and ass in a paper in which they report on the influence of temperature and composition on the viscosity of such milks. Other workers from India(29) have studied the viscosity of cow and buffalo milk, and have shown(30) that viscosity increases to about twice the original value, when 10 ml. of strong ammonia, e.g. 2N, are added to 90 ml. of milk. The final value depends on the protein content of milk, and may be used to calculate total protein with results shown to be in fair agreement with those obtained by Kjeldahl determinations. Data on the dynamic and kinematic viscosity of goat's milk are to be found in (31). Factors affecting the viscosity and whipping properties of cream have been discussed in detail by the late H. H. Sommer(32). Perhaps the most surprising observation made by Yugoslav workers(33) who studied the changes in viscosity induced by souring organisms and rennet with the help of a Hoeppler viscometer, is that the viscosity of milk tended to fall for as long as 15 min. after rennet addition at 30° C.

(*e*) *Freezing.* Babcock and his associates, protagonists in the field of frozen storage of homogenized milk, have continued their work by giving attention to the distribution of the main constituents of milk during and after freezing at −20° C.(34). With homogenized milk, the butterfat tended to accumulate in the bottom portion of quart-samples, whilst the top portion was richest in fat with milk which had not been homogenized. A useful, brief summary of their whole series of studies is to be found in(35). The advantages of pasteurization and homogenization of milk intended for frozen storage are stressed by Samuelsson(36) who, like the American workers, recommends rapid freezing. Rose & Tessier(37) found the rate of freezing to be of little significance with milks which were to be stored at −18° C., but obtained the best results with *slow* freezing when milk was to be held at −12° C. Keeping quality was judged by measuring the volume of centrifuged casein precipitate in thawed samples according to a procedure laid down by Rose(38) who considers it adequate for rapid testing, though more precise results can be obtained by determining the nitrogen content of the supernatants of the centrifuged protein. Methods based on protein precipitation, also used by Babcock and his colleagues, may be of some practical value in assessing the keeping properties of frozen milks, but as the apparent destabilization of casein is often largely reversible when such milks are held at blood-heat (about 40° C.) for a short period, one would wish for the development of methods, at least for research purposes, that take this remarkable fact into consideration.

In a later series of papers(39,40,41,42), Rose and his colleagues consider the influence of various factors, such as preheating conditions, on the keeping properties of frozen milk, stressing, in particular, the destabilizing effects caused by lactose crystallization(39,40). The adverse influence on casein stability of the formation of lactose crystals had also been demonstrated earlier by Tumerman, Fram & Cornely(43) who, working with concentrated milk, were able to demonstrate that such milk had improved properties after frozen storage at −10° C. when part of the lactose had been removed by crystallization or hydrolysis. Their paper contains interesting data on the proportion of the total water content of the milk concentrates found, by a calorimetric procedure, to be frozen at temperatures varying from −3 to −28° C., the proportion increasing from 22 to 94·5% over this range.

Preservation of cow's milk by freeze-drying on a large scale is not, at present economic. That this gentle process may be of considerable value in the conservation of relatively small volumes of human milk is stressed in three German publications(44,45,46). Nikolić(47) recommends it with enthusiasm for products such as yoghurt and kefir, and goes so far as to describe the layout of a suitable large-scale plant.

(2) ADULTERATION AND ITS DETECTION

(*a*) *Added water*

(i) *The freezing-point test.* Since this subject was last reviewed(48), increased attention has been given to this test in the U.S.A. At the 1953 meeting of the Association of Official Agricultural Chemists, Dubin(49) pointed out that doubts had arisen about the reproducibility of the Hortvet

technique as adopted by the Association and about the fairness of the method of interpretation based on a tolerance of 3% on an assumed mean freezing-point depression (Δ) of 0·550° C., there being evidence that this assumed value was too great and the tolerance, as a consequence, inadequate. Concerning lack of reproducibility, attention was drawn to a report by Shipe, Dahlberg & Herrington (50) that nine collaborating laboratories obtained widely varying results when testing samples of the same milk, e.g. Δ-values for a genuine milk ranging from 0·535 to 0·555° C. In their own laboratory, workers produced much more uniform results when they used an automatic cooling bath instead of the Hortvet cryoscope, and mechanical devices to stir the milk and to tap the thermometer (51). The latter device consists of a rubber mallet impinging vertically on the top of the thermometer at the rate of 100 strokes per minute. This seems excessive, as a few gentle taps, logically applied at the level of the top of the mercury column, suffice to overcome sticking of the mercury.

Using the modified cryoscope, Shipe *et al.* (50) obtained a mean of 0·539° C. for 594 genuine milk samples. Dubin (49) refers to these results and also to data given by Dahlberg, Adams & Held (52), and later discussed by Dahlberg (53), as indicating that an assumed mean of 0·550° C. is untenable. These authors found that pasteurized bulk milk supplies in eight large cities varied in Δ from 0·535 to 0·553° C., and concluded that there were significant differences from one area to another. This evidence is hardly convincing, since the variations may be no more than a reflexion of the different amounts of extraneous water that have gained access between the times at which the milks left the cows and the processing plants.

On the basis of this and other evidence, Dubin proposed that further work should be done by the A.O.A.C. to ensure greater uniformity of testing, and that such work should then be followed by a survey of genuine herd milks designed to arrive at a more reliable and realistic mean Δ-value. Unfortunately, this course has not been adopted, and the survey (54,55,56) has been carried out without elimination of discrepancies between the results of the collaborating laboratories. As shown in the second part of the survey report (55), replication between laboratories was poor, and this makes it difficult to interpret the results for 1627 herd milks (mean Δ: 0·543° C.) which, taken at their face value, imply that there are marked regional differences throughout the U.S.A. Whether these differences are of sufficient magnitude to consider regional standards of interpretation, as has been suggested, can hardly be decided before more precise results come to hand. That greater precision is attainable has been shown by collaborative work in Britain (Aschaffenburg & King (1951), *Analyst*, **76**, 2) in which participants adhered to a closely defined technique, and made use of a more reliable method of thermometer calibration than that of Hortvet which does not eliminate errors due to inequalities in the bore of thermometers. Meanwhile, the interpretation of a test of undoubted value is likely to remain in a state of uncertainty in the U.S.A.

What makes one sceptical about the reality of regional differences of freezing-point distributions within the confines of the U.S.A., are observations suggesting that mean Δ-values do not differ significantly from one part of the globe to another. Thus, Indian workers (57) report a value of 0·545° C. for some 200 samples collected from all parts of their huge country. For the buffalo, the corresponding value was 0·544° C. Greater depressions were found for goat's milk (0·579° C.), and ewe's milk (0·587° C.) (58). Elevated Δ-values were observed in the colostrum of these species (58,59), and the increases could not be accounted for in terms of lactose and chloride. In agreement with others, the Indian workers found pasteurization to be without effect (59), whilst Pinkerton & Peters (60) claim that H.T.S.T. treatment leads to decreases in Δ (no figures given). Applying the Hortvet technique to human milk, Miller & Ellis (61) obtained a mean of 0·586° C. Samples from the two breasts were often very similar, and did not differ by more than 0·01° C.

Williams & Ellis (62,63) and Houlbrooke (64) have presented fresh cases in which unbalanced intake of drinking water by the cows of a herd resulted in the secretion of evening milk with unusually low Δ-values and, often, of morning milk with unusually high Δ-values. Evening values of less than 0·530° C. were quite common. The cases described by Williams & Ellis are of

particular interest, in that it was possible to balance the water intake, and to demonstrate the resulting return to normal freezing-points, though on one farm (63) it proved difficult to wean the cattle from the habit of drinking water but once a day. All these cases were observed in late winter. The bearing of such, apparently rare, occurrences on the detection of added water by the freezing-point test, is discussed in (65). An instance of low Δ-values for the milk of cows of a herd has also been reported from Texas (66). Whether this was of a similar nature to the cases discussed above, cannot be decided, as the report contains no information about the watering regime of the cattle and about the nature of the milk samples (evening milk?). It is, however, perhaps significant that the observations were made in late winter, and during a period of drought. A distinctly different kind of case, in which unbalanced water intake could be ruled out, has been described by Bagnall & Smith (67). Abnormal Δ-values down to 0·518° C. may here have resulted from the transfer of the cows to spring pasture.

Chindemi (68) has reviewed freezing-point techniques currently used in various countries. Promising results, rapidly obtainable with small volumes of milk, have been given by an apparatus—presumably the 'Fiske osmometer'—using thermistors in place of the mercury thermometer (69). Polish (70) and Australian (71, 72) authors have given consideration to the relationship between lactose, chloride and the freezing-point of milk.

(ii) *Other methods.* Methods for the detection of added water other than the freezing-point test have, in general, proved disappointing, and attempts to develop such methods seem to have ceased almost completely. Curdled milk presents a problem, as the freezing-point test is no longer of use. Effern has examined a number of recommended procedures (73) of which determination of the ash content gave the most reliable results. As a quick method, he has later recommended the freezing-point value calculated from the refraction of the lead serum and the chloride content of milk (74). All his work refers to large additions of added water, e.g. 10, 20% or more. Detection of adulteration of buttermilk presents another special problem, and methods proposed for its solution have been critically examined by Kiermeier & Pirner (75) who consider determination of ash the method of choice for stale buttermilk, and recommend a rapid densitometric technique (Schulz & Digeser (1937) *Molkereiztg, Hildesh.* **51**, 781) for serial testing of fresh samples.

(*b*) *Other types of adulteration*

Butterfat. During the period under review research into methods designed to detect the presence of foreign fats in butter has been done almost exclusively in two countries, the U.S.A. where adulteration with both animal and vegetable fats appears to be of concern, and Italy where adulteration with hydrogenated dolphin oil presents the main problem. The reviewer, not being familiar with the numerous testing procedures, can do no better than draw the reader's attention to a recent American review by Bhalerao & Kummerow (76) who present a critical discussion of a variety of techniques, and show at the hand of experimental data that a method perfected in their own laboratory (77), and based on the typical glyceride structure of butterfat, could detect the presence of a greater variety of foreign fats at the 10% level than the other procedures examined. Favourable comment on one of the methods has come from Holland, where Den Herder (78) studied the sterol acetate test and found it satisfactory for detecting adulteration with vegetable fats.

Information on Italian methods is contained in a chapter of Neri's book on butter, published in 1954 (79). Amongst the techniques described is the simple Tortelli–Jaffe colour reaction. According to Cerutti (80) who applied this test to a variety of fats and oils, only sesame oil gives a yellow colour like butter. Mahon & Chapman (81) also report favourably on this test as a means of detecting the presence of fats derived from marine animals, but caution is advocated by Curli (82) who found that various colours may develop when butter contains additives such as colouring matters or antioxidants. That the test is not specific for fish oils has been pointed out by Macciotta & Mannironi (83). They obtained positive reactions with the butterfat of ewes' milk which also reacts differently from ordinary butter in another established method, Bellier's resorcin test.

This test is discussed by Francesco (84,85,86) who has developed another technique based on the critical temperature of solubility of the non-volatile fatty acids in nitrobenzene.

Useful as these techniques may be, the method of choice that is emerging from the attempts to solve the problem of adulteration of butter with hydrogenated dolphin oil appears to be the more specific one of detecting the presence of *iso*valeric acid. A variety of techniques designed for this purpose has been described, based on distillation of the water-soluble volatile fatty acids, followed by titration (87), partition chromatography on a silica gel column (88) or ascending paper chromatography using different solvent mixtures (89,90,91,92,93). According to Chioffi (94), the Reichert–Meissl number, and the specific electrical conductivity of the distillate prepared for the determination of this number are linearly related in genuine butter, and the relationship is changed by the presence of *iso*valeric acid. That the determination of *iso*oleic acid may also be utilized for the detection of butter adulteration with hydrogenated fish oils, has been shown by Cerutti (95). Identification of triacetin has also been suggested as a possibility (96).

To detect, and to determine quantitatively, various colouring agents added to butter or margarine, Espoy & Barnett (97) have evolved a scheme involving column chromatography of solutions in hexane and subsequent spectrophotometric analysis.

Miscellaneous. Several colorimetric methods for the detection of additions of reconstituted milk to pasteurized milk have been described (98,99,100,101), all based on colour-forming reactions of the isolated curd. The presence of reconstituted spray-dried milk powder in raw milk can be demonstrated in a simple manner according to Vallier (102). Very small additions of sucrose to milk can be easily revealed by simple colorimetric tests (103,104), or by circular paper chromatography (105) which seems less sensitive. According to Italian workers (106), the pH of milk increases considerably on boiling when bicarbonate has been used as a neutralizer, e.g. by 0·4 units for additions of 0·1 %. An iodine-starch procedure is recommended by French authors (107) to detect the presence of hydrogen peroxide in milk.

Additions of cow's milk to human milk can be revealed in several ways by the preferential flocculation of the casein of cow's milk in acid media. Mixtures of acetic acid and either alum or alcohol may be used (108) or, as first suggested by Freudenberg (1939, *Schweiz. med. Wschr.* **69**, 515), an acetate buffer containing calcium chloride, a procedure which Flies (109) found sufficiently sensitive to detect additions of 4 % of cow's milk. Differential formation of crystals in drops observed under the microscope, a technique named 'stagoscopy' by Solé (110), is said to be useful for the same purpose.

Detailed procedures for the detection and determination of gums in dairy products are given in (111,112,113).

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PART II. MILK COMPOSITION AND ANALYSIS, AND CHEESE RIPENING

A perusal of work published since the last review has led the writer to the conclusion that this survey should concentrate upon two subjects only—milk composition and analysis, and cheese ripening. If such a choice requires justification, the writer would plead that the published work has impressed him more in these two directions than in the other ramifications of Dairy Chemistry.

(1) MILK, ITS COMPOSITION AND ANALYSIS

From time to time attempts have been made to correlate the percentages of various milk constituents, and the tendency just now is to examine the fat/protein relationship. Vanschoubroek & Willems (1) consider it most probable that the regression of protein on fat is genetically determined. Data for 218 cows of the same breed, fed and managed under identical conditions, furnished the regression equations $Y = 2{\cdot}4111 + 0{\cdot}1644X_1 + 0{\cdot}00053X_2$, where Y = percentage protein, X_1 the percentage fat and X_2 the number of litres of milk; or neglecting the volume of milk

$$Y = 2{\cdot}424 + 0{\cdot}164X_1.$$

Similarly, Marckmann & Hingst (2) observed that fat was significantly correlated with protein in milk from 1500 cows in Schleswig–Holstein. In the majority of cases the correlation coefficient ranged from +0·6852 to +0·5837. Observations were spread over a complete year. While fat percentage was higher in winter than in summer the protein content showed a contrary tendency and did not seem to be influenced by feeding. Minimum protein levels occurred in April but a decline also occurred in November. A slight fall in June–July coincided with a fall in fat percentage. Both fat and protein were highest in the second and third lactations, beyond which protein declined steadily, whereas the fat was relatively constant until the tenth lactation. The question naturally arises whether the fat-protein correlation concerns all the protein constituents. Vleeschauwer & Heyndrickx (3) found significant correlation between fat and total protein; and total casein but beyond this, the results were inconclusive. Jenkins & Provan (4) consider that under similar conditions of management a rise in fat percentage will be accompanied by an increase in non-fatty solids, and that it is still seasonable to assess the attainable S.N.F. level of the milk of a herd or group of cows by the fat percentage.

A comprehensive investigation by the Milk Marketing Board (5) into the composition of milk from six Friesian and three Shorthorn herds during the period 1948–52, showed that the herd averages ranged from 3·21 to 3·66 % fat and 8·52–8·87 % non-fatty solids. From the considerable weight of evidence, the investigators concluded that to avoid occasions on which the milk of the herd contains less than 3 % fat and 8·5 % S.N.F. the herd average must be kept above 3·4 % fat and 8·7 % S.N.F. This may not be possible if the herd contains many cows in their fourth or later lactations, or if the protein or energy levels of the ration are not adequate. In this work the non-fatty solids declined by 0·1 % in each succeeding lactation. Similar observations on the latter effect were made by Griffiths & Featherstone (6), but Waite, White & Robertson (7) found that beyond the third lactation, the rate of decline was not so great. They found the differences between the first and ninth lactations were 0·19 % fat, 0·35 % S.N.F., 0·21 % casein and 0·25 % lactose. It is very significant that, in the work of Griffiths & Featherstone (6) the feeding of extra starch equivalent to cows giving milk of low non-fatty solid content, produced no improvement in that direction.

A series of papers from the Hannah Dairy Research Institute have reopened the question of milk composition as influenced by age, stage of lactation, season (7), heredity (8) and winter feeding (9, 10, 11). These subjects have claimed the attention of research workers at intervals since the commencement of the present century, but the changing conditions of agriculture and milk production, improved methods of analysis and the greater emphasis at present laid upon the non-fatty solids of milk make it highly desirable to re-examine the whole subject. The investigation covering the period 1950–53 involved the sampling and analysis of milk from 814 Ayrshire cows at intervals of approximately 5 weeks. It was observed that the effects of season were largely dominated by lactation effects and the authors were able to arrive at true values only after applying lactation corrections. They followed the general trends which we have come to expect—but the variations in lactose content followed the general trend of yield, thus moving in the opposite direction to fat and casein. On the 45th day of lactation the yield reached a maximum but the concentration of non-fatty solids, casein, and total protein reached their minima. Fat, and therefore total solids reached their minima on the 75th day. Lactose concentration moved

with the yield and suffered a more abrupt decline after the 165th day. This decline was much more severe with older cows. The fall in fat content at the commencement of grazing was considered more likely to be due to the effects of the sudden change in the nature of the rumen contents rather than deficiencies of fibre in the grass. Of the total number of individual samples, 2% were below 3% fat and 16% below 8·5% S.N.F., and the most critical periods of the year were January–April and to a lesser degree July–October for non-fatty solids; and May for fat percentage. The authors point out that cows calving from mid-November to the end of February will reach the lactational minimum run of non-fatty solids at the critical period when they may suffer a decline from other causes. The conjunction of these two factors, particularly if aggravated by a high proportion of cows past their third lactation, may well be responsible for much of the trouble with low-quality milk at this time. On the influence of heredity (8) the authors note that cows which genetically tend to produce milk with high casein concentration will also tend to give milk above average in fat and lactose content. They concluded that selection for yield alone was, of itself, unlikely to cause any great decline in non-fatty solid content, and that any such tendency would probably be kept in check by paying attention to the fat content. While these authors concluded that the genetic correlation between fat and lactose concentration was almost significant, Vanschonbroek & Willems (12) found a positive regression of lactose on fat which was genetically determined.

High-plane nutrition experiments on the winter feeding of twelve Ayrshire cows (9) furnished the observation that when the concentrate contained three levels of starch equivalent 59, 67 and 75%, and a constant protein (16% digestible crude protein), the higher starch equivalent feeds gave significantly more milk and a tendency towards an increased live weight. In the 67% group the level of non-fatty solids in the milk was raised somewhat, due mainly to an increase in protein content. No further benefit was to be obtained by maintaining a steady level of 63·5% starch equivalent in the concentrate, and increasing the digestible crude protein from 10–15 and 17%. A comparison between the feeding value of dried spring grass and dried autumn grass showed that the yield was some 3–5% higher with the spring grass (10). The figures were, however, considered not to be statistically significant. The third paper (11) on winter feeding brought the studies to their logical conclusion in the feeding of eight Ayrshire cows on a diet composed of high-quality dried grass and silage, together with a high- or low-protein concentrate at the rate of 2 or 4 lb./gal. yield. For every 1 lb. increase in concentrate fed there was a decrease of 0·68 lb. grass dry matter consumed. From the observed increases in milk yield, the authors concluded that the greatest margin of profit per cow was achieved by the addition of 2 lb./gal. of concentrate mixture, whether of high- or low-protein content being immaterial. The influence of feeding during lactation on the yield and composition of milk is the subject of a very comprehensive review by Burt (13).

Very marked seasonal variations in the citric-acid content of bulked skim-milk delivered to factories in Friesland have been reported by Holwerda (14). Lowest values 0·131–0·138% were found in November. A peak value in April 0·173% declined somewhat to 0·169% in October. The abrupt decline in November did not necessarily coincide with the change from outdoor to stall feeding. Low values were found to be associated with late lactation milk.

It has been customary to consider that variations in the composition of milk during the course of milking concern the fat content only, but Gardner, Thomas & Willard (15) found that calculated on a fat-free basis, there is a higher level of S.N.F. in the middle portion. Typical figures were 2·4, 3·8 and 8·9% fat, and 8·3, 8·6 and 8·2% S.N.F. in the first drawn middle portion, and strippings, respectively. Further, to a previous observation that disease causing a rise in body temperature above 102·4° F. brings about low S.N.F. and high fat percentage in milk, King (16) observed that when the disease does not cause the body temperature to exceed this value the milk shows no such tendencies. Although recovery in these cases caused an increase in yield, neither fat nor non-fatty solids were affected. Reinart & Nesbitt (17) found that the nitrogen distribution of milk in Manitoba varied with the breed. Samples were taken from twelve pure-bred herds and bulk samples from three of the largest dairies in Winnipeg (Table 1).

Table 1. *Nitrogen distribution as percentage total N, (average values)*

	Holstein	Jersey	Ayrshire	Guernsey	Brown Swiss	Red Poll	Bulk
No. of samples	75	72	70	23	23	20	51
Casein N	77·7	79·0	77·4	77·9	77·8	80·0	77·0
Albumin + globulin N	12·7	12·9	12·4	12·5	12·4	11·0	12·2
Proteose-peptone N	4·0	3·7	4·9	4·1	3·8	3·9	4·8
Non-protein N	5·7	4·4	5·3	5·5	6·0	5·2	6·0
Percentage total nitrogen mg./100 ml.	521·5	622·1	562·2	600·8	549·8	580·7	514·7

The general composition, including Ca, P, Mg, Cl was also determined (18), some of the figures being shown in Table 2.

Table 2

	T.S. (%)	S.N.F. (%)	Fat (%)	Protein (%)	Ash (%)	No. of samples
Holstein	11·91	8·35	3·56	3·05	0·73	75
Jersey	14·15	9·19	4·97	3·66	0·77	72
Ayrshire	12·69	8·72	3·97	3·25	0·72	70
Guernsey	13·69	9·14	4·58	3·50	0·75	23
Brown Swiss	12·69	8·89	3·80	3·18	0·72	23
Red Poll	13·28	9·05	4·24	3·40	0·72	20
Bulk	11·78	8·34	3·44	3·02	0·73	68

Apart from fat percentage, in all herds the coefficient of variation was greatest for chloride and magnesium and least for ash and non-fatty solids. The carotene and total vitamin A potency of the fats were also determined (19).

Methyl sulphide is considered to make a contribution to milk flavour (20). Increasing concentrations impart flavours described as 'milky', 'malty', 'cowy or feedy'. The gas was identified in the breath of the cow and in the gases removed by passing air through raw milk in a 1000 gal. cold-wall tank.

With the acid of dinitro-fluorbenzene which couples with free-amino groups, it is possible to follow the removal of lysine which occurs when skim-milk is heated at 100–125° C. for periods up to 20 min. Spray and roller drying had no appreciable effect on lysine content (21). However, Mauron, Mottu, Bujard & Egli (22) did observe slight losses on spray drying (3·6%), rather more in roller drying (13%) and that scorching in roller drying caused a loss of 26·6%. Losses for sweetened condensed milk were 4·8%, and for evaporated milk 8·4%. Tryptophane, tyrosine, methionine, and α-amino groups were not affected, except in one sample of slightly scorched roller-dried powder. No losses of lysine occurred on boiling milk. Where this amino-acid was lost, it was considered due to the sugar-amino reaction.

During the period under review papers have appeared on the composition of milk from the following: Tagil (23), Red Steppe (24), Aulie-Ata (25), Alatau (26), Kurgan (27) and Armenian cows (28). Russian breeds of cattle, zebu, yaks, buffaloes and cross-breds (29). Egyptian buffalo (30,31,32), goat (33,34) and Polish ewes (35,36).

The tri- and tetra-saccharides in human milk continue to receive attention. Two isomers of a tri-saccharide were found to contain one molecule each of glucose, galactose, and fucose (37,38,39), and a tetra-saccharide was also present containing one molecule each of galactose and glucose, and two molecules of fucose (40). Glycoproteins have been observed in the milk of various species (41,42,43). Johansson & Svennerholm (42) determined the carbohydrates, etc., in caseins prepared from the species shown in Table 3. The physiological importance of these carbohydrate fractions is at present unknown. Stary & Cindi (43) determined the protein-bound carbohydrate of seven differently prepared caseins. The chief carbohydrate was galactose. They observed that other proteins of milk also contained carbohydrates.

Table 3. *As percentage of dry casein*

Species	Total carbohydrate	Hexosamine	Hexose	Sialic acid
Human	4·03	1·32	1·98	0·76
Cow	0·80	0·18	0·24	0·39
Ewe	0·55	0·15	0·23	0·11
Goat	0·68	0·16	0·22	0·30
Whale	1·38	0·42	0·59	0·37
Mare	1·55	0·44	0·55	0·56
Reindeer	1·13	0·23	0·44	0·46

Milk analysis

Preservation. The use of potassium dichromate is considered by Pietz(44) to cause difficulties in the Gerber test in that the curd may be difficult to dissolve. A 2% solution of Thiobaktal (sodium-methyl-mercurithiosalicylate) added at the rate of 0·25/50 ml. milk overcame these difficulties. Bejambes, Mocquot & Pauthe(45) compared the relative merits of potassium dichromate, mercury-phenyl-borate and formaldehyde acting in conjunction with tri-oxymethylene. They concluded that the most satisfactory treatment was the addition of 0·6 ml. of commercial formalin and 0·4 g. tri-oxymethylene per litre of milk. Bacterial numbers declined rapidly and over a 50-day test lactose concentration was unchanged.

The increasing appreciation of the value of the non-fatty solids of milk has intensified efforts to improve existing methods for their determination or to discover new ones. Leggatt, Dinsmore & Sproule(46) have improved their ceric sulphate titration method to bring it within −0·24 and +0·21% of the gravimetric determination. The density or specific gravity formula methods have been the subject of several researches. From observations on 634 samples, Pascoe(47) concluded that the present B.S.I. formula should be amended to

$$\text{T.S.} = 1{\cdot}25F + 0{\cdot}265D + 0{\cdot}11.$$

The fat was determined by the Babcock method and the gravimetric method was used to check the accuracy of the formula. The B.S.I. method involves warming the sample to 40° C. to eliminate the Recknagel effect before reading the density at 20° C. It would obviously be quicker and more convenient to take the density at 40° C. and make suitable amendments to the formula. Such a procedure would necessitate the use of a specially calibrated hydrometer. This was the approach to the problem made by Watson(48). The sample is warmed to 102° F. in a thermostatically controlled water-bath, and the specific gravity determined at that temperature, using a hydrometer graduated at 102° F. from 1·0256 to 1·0374, each hydrometer degree being subdivided to read 0·2 degrees (i.e. 0·0002 sp.gr.). Using the Babcock method for the fat content, Watson established the formula

$$\text{T.S.} = 1{\cdot}254F + 0{\cdot}271L - 0{\cdot}216, \quad \text{where } L = 1000\,(\text{sp.gr.} - 1) \text{ at } 102° \text{ F.}$$

95% of the 101 bulk milk samples examined gave values which differed from the gravimetric method within the range −0·19 to +0·18% T.S.

The B.S.I. recommendations for the Gerber test(49,50) have been amended, the chief alteration being a reduction in the capacity of the milk pipette from 11·04 to 10·94 ml. in accordance with the findings of Crocker *et al.*(51). The attraction of a non-acid fat test is again apparent from the number of papers which have recently appeared. Various reagents have been used to break the fat emulsion, for example: (*a*) sodium hydroxide, sodium citrate, sodium salicylate, ethyl and isobutyl alcohols(52); (*b*) borax, amyl and ethyl alcohols(53); (*c*) detergents with(54) or without other reagents(55). Other methods are solvent extraction after dehydration with anhydrous sodium sulphate(56) or calcined soda ash(57), and a micro method involving the use of hydroxylamine(58). The latter depends on the formation of hydroxamic acid by reaction with the fat and the subsequent colorimetric determination of this compound as red ferric hydroxamate.

Volatile acids in milk have been determined by the gas chromatographic method(59) and in homogenized milk by distillation and titration(60). The pressure and temperature affected the

values, which normally ranged from 1–4 ml. N/10 NaOH/100 g. milk. Total free fatty acids of milk were determined by a rapid silica-gel method (61) and by extraction with 40% ethyl ether and 60% petroleum 'ether' followed by titration with alcoholic potassium-hydroxide (62).

Three methods for the determination of lactose have appeared, one an adaptation of the method of Benedict & Osterberg (Knaut (63)), one using a ferric chloride oxidation of the casein-free serum (64), and the third employing the polarimeter with a zinc acetate–phosphotungstic acid–glacial acetic acid serum (65).

Marier & Boulet (66) put forward a turbidometric method for the micro-determination of calcium in milk. Measured quantities of citrate-phosphate and gelatin-oleate reagents were added and the extinction coefficient determined at 420 mμ some 20–60 min. after swirling the mixture. Raadsveld (67) titrated the calcium in a diluted sample of milk with versenate (ethylenediamine tetra-acetic acid-disodium salt), claiming that phosphates do not interfere. Murthy & Whitney (68) however, remove phosphates from a hydrochloric acid serum by passing through an anion exchange resin, and then determine all the major cations by the flame photometer and versenate titration methods. Methods for the determination of sodium (69), potassium (70), cadmium (71), copper (72), sulphate (73), iron and copper (74) have been published during the period under review.

A promising approach to the problem of determination of lipase activity was the use of esters of 2-naphthol-6-sulphonic acid (sodium salt) as substrate. (75) The butyryl or caprylyl esters are sufficiently water soluble for this purpose. Folin & Ciocolteu's reagent is used to determine 2-naphthol-sulphonic acid liberated by lipase.

An assessment of the contributions of lactose and chloride to the freezing-point depression of milk was made by Cole & Mead (76) who found that 5% lactose hydrate contributes 0·294° C. and 0·1% chlorine as chloride 0·110° C. A 44-page report by Robertson (77) deals with the distribution of freezing-point depression among 1627 herd milks drawn from many different areas of the United States of America during the period 1954–6. While the mean of all samples was 0·543° C. those for separate areas varied from 0·528 to 0·549° C. It was suggested that each area should decide upon its own minimum freezing-point depression for the purpose of detecting watering. In collaborative tests it was evident that strict adherance to details of apparatus and technique were essential. Although in one instance the operator was successful in detecting 1·5% added water, all his values showed an error of 0·01° C. because of faulty, yet in this case, consistently faulty, technique. In its passage from the farm to the consumer, Sato, Hankinson, Gould & Armstrong (78) found milk to undergo the changes shown in Table 4.

Table 4

	Freezing-point depression			No. of samples
	Min.	Max.	Mean	
Individual or herd samples	0·533	0·560	0·546	59
Producers' milk received at dairy	0·450	0·550	0·538	108
From dairy storage tanks	0·523	0·541	0·533	10
Retail samples	0·437	0·549	0·530	53

The authors suggest that 0·530° C. represents a reasonable basis for discriminating between genuine and watered samples. Bagnall & Smith (79) reported an instance of genuine milk from thirty-two cows (nineteen of which were in early lactation) giving freezing-point depressions of 0·518° C. a.m. and 0·522° C. p.m. The samples were taken when the cows were only recently turned out to grass.

With the recognition that in many cases fluctuations in the non-fatty solid content of milk is caused by variations in protein concentration there has been a welcome enquiry into methods for its determination. Dye adsorbtion by milk proteins offers attractive possibilities. Amido-black at pH 2·8 was used by Schober & Hetzel (80), the surplus dye being measured by the extinction at 578 mμ. Udy (81) used Orange-G at pH 2·2, and measured the excess dye after shaking and centifuging. While Schulz, Vos & Kay (82) found the formol titration a rapid and convenient routine method, Bosticco (83) observed that divergence from the Kjehdahl values was appreciable

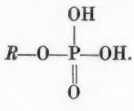
in early and late lactation, in milk which has suffered proteolysis, and in milk from cows in ill health. The direct distillation, in which milk is distilled with caustic soda for an arbitrarily selected length of time, has been found by Antila(84) to be more reliable than the formol method, the errors on individual samples being ±5·2% formol method and ±4·7 direct distillation—taking the Kjeldahl method as standard. A statistical analysis of data from fifty samples of cows' milk, seventy-three of goats' milk and fifty-two of ewes' milk, led Neseni & Schaaf(85) to the conclusion that the direct distillation procedure was sufficiently accurate for routine testing. Waddell(86) has described a simple rapid ultra-violet spectrophotometric method which is more sensitive than the Kjeldahl procedure.

(2) *Cheese*

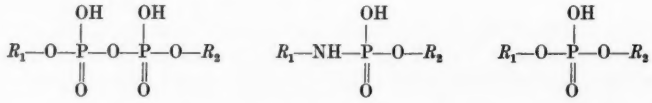
Most of the work during the period under review has been concerned with the ripening process. These studies are valuable not only in their bearing upon cheese making, but in so far as they are able to reveal much of the chemical nature of the major constituents. Further, the extremely wide range of chemical, physical and microbiological conditions pertaining to the many varieties of cheese gives added confidence in the validity of any conclusions which may be drawn.

There are five lines of approach: (*a*) amino-acid production; (*b*) acids other than amino-acids; (*c*) carbonyl compounds; (*d*) other compounds; (*e*) flavour production. As a result of chromatographic studies, a great mass of information is now available for many varieties of cheese concerning the number, nature, and quantities of amino-acids which may be expected at various stages of ripening. It is a common experience that the concentration of any one amino-acid in cheese of the same age and variety shows wide variation even when made in the same factory. The study is also complicated by the fact that many of the liberated amino-acids are subject to further fermentative changes.

In a comprehensive study of the ripening of acid skim-milk cheese, Schormüller(87,88,89,90,91) discovered that while the ratio P/N remained constant, there was an increase in the amount of acid soluble P and N particularly in the later stages. Furthermore, the quantity of inorganic phosphate showed an increase(91). These observations were confirmed by Klimovskiĭ, Tikhamirova, Chekalova & Shlyapnikova(92) who found that with a much slower ripening rennet cheese there was an increase in organic phosphate as ripening advanced. The researches of Perlmann(93) have established that the greater part of the 'ester' phosphoric acid of acid-precipitated casein is used to cross-link adjacent polypeptide chains or to link parts of the same chain or coil. Only a small proportion, 40% in α-casein and none in β-casein exists in the form



The cross-linkages may be of the following types:



When the peptide chains are broken by hydrolysis, phospho-peptones or phospho-amino-acids may appear; but only after the cross-linkages are broken will the maximum quantity of phosphorus appear in the products of ripening. It seems to the writer that further investigations along these lines would provide valuable information. Serine and Threonine, two hydroxy amino-acids known to unite with phosphoric acid through the hydroxy group, were observed by Schormüller, Glathe & Huth(94) to pass first into the water-soluble protein phase and ultimately into the non-protein nitrogen. They were also further fermented to α-alanine and α-keto-butyric acid and α-aminobutyric acid.

If as it is claimed the terminal amino-acids of both α- and β-casein are arginine and lysine it is of interest to enquire at what stage of the ripening process these acids are set free. The inquiry is the more pertinent in view of the fact that the more probable —NH—P— cross-linkage in α-casein is phosphoarginine. In an examination of thirty Cheddar cheese Bullock & Irvine(95) observed that while lysine appeared quite early in the ripening period, arginine was detected only after 60 days with the raw milk cheese, and 130 days with those made from pasteurized milk. While it is dangerous to come to a conclusion from the evidence of one investigation it would seem that, whether due to phosphoric acid or not, the arginine region of the casein molecule may be rather more resistant to attack by the hydrolytic agents in Cheddar cheese. Other compounds making a late appearance were histidine (170 days), proline (180 days) and tyramine (6 months in raw milk cheese). Phenylalanine, serine, alanine, aspartic acid, and threonine increased in concentration to a steady level, but valine, lysine, leucine, methionine and glutamic acid concentrations increased steadily with age. Inoculation of the cheese-milk with *Lactobacillus casei* had little obvious effect up to 4 months storage, but at 8½ months it was evident that amino-acid release was greater in this cheese than in the uninoculated control.

Tyrosine made its appearance quite regularly during the ripening of Cheddar cheese(96). As much as 6% of the combined tyrosine of the cheese protein was liberated in 176 days at 60° F. It may be converted to tyramine by decarboxylases. After the initial stages, tyrosine liberation outpaced the formation of soluble protein and the authors suggest that the concentration of this amino-acid gives a better indication of the stage of ripening than does the ratio soluble/total nitrogen. Total free-tyrosine concentrations ranged from 1341 to 3583 p.p.m. In this work it was evident that tyrosine was being detached from the constituents of the water-soluble phase, but there is still a possibility that, in addition, it may be detached directly from the paracasein molecule.

The course of ripening of New Zealand Cheddar cheese made from pasteurized milk was reported by Dacre(97). The work is noteworthy in that the time of appearance of fifteen amino-acids has been noted. Although in the cheese examined their rate of appearance varied, it is possible to group them in a general order of appearance.

(1) Glutamic acid, lysine or arginine, leucine and iso-leucine, tyrosine.
(2) Valine, alanine, phenylalanine.
(3) Aspartic acid and glycine.
(4) Glutamine and asparagine.
(5) Methionine.
(6) Threonine, serine, proline.

In this work the two basic amino-acids have been determined together; but it would appear from the work of Bullock & Irvine(95) that only lysine makes an early appearance. Further investigation is clearly needed to settle this matter. The concentration of all amino-acids increased steadily during ripening except tyrosine, which is decarboxylated by *L. brevis* strains normally present in New Zealand Cheddar. Small amounts of a soluble peptide also appeared after 2 months ripening. It is interesting to note that in the previous review, the work of Jacquet & Lenoir(98) with Camembert cheese was mentioned. In this very different kind of cheese glutamic acid and leucine appeared very early indeed, followed by alanine, arginine, lysine, histidine, tyrosine, and valine, and lastly by glutamine, methionine, phenylalanine, proline and oxyproline. Future work will perhaps show whether for any variety of cheese there is a general order of appearance of amino-acids.

Mabbitt's work on Cheddar cheese(99), also mentioned in the previous review, emphasized the difference in amino-acid pattern between the ripened portion of cheese protein and casein. In the liberated amino-acids, the ratio basic/acidic constituents was greater than in casein. Proline which is present in casein to the extent of 13%, constituted only 4–5% of the free amino-acids of a 6 months ripened Cheddar. Ornithine and cysteic acid are not constituents of casein, but appeared in the ripened portion of the Cheddar cheese. The cysteic acid fraction contained unidentified material, and quite a number of unidentified peptide fractions made their appearance

during ripening. The probability that in Swiss cheese, glutamic acid is converted to proline during ripening is suggested by the work of Hintz, Slatter & Harper (100). These workers identified a cysteic-acid fraction, but being in excess of what might be expected from casein, they concluded that other material was present. With this type of cheese as with Cheddar, the ratio basic/acidic-free amino-acids was found to be higher than in casein (101), 41 % being neutral, 38 % basic and 21 % acidic.

According to Gonashvili (102), the increase in non-protein nitrogen in ripening Soviet cheese is more rapid than that of soluble protein nitrogen, leading to the conclusion that amino-acids may be detached directly from the casein molecule as well as from the soluble proteoses. At 4 months, some 77 % of all amino nitrogen of the polypeptide amino-acid fraction was in the form of amino-acids. In Moravian loaf (hard) cheese (103) amino-acids predominated over albumoses and peptones even in the first few weeks of ripening. Those of small molecular weight were particularly vulnerable to further fermentation.

The relative increase in the basic amino-acids of the ripened cheese protein raises many questions. It remains to be seen whether this circumstance arises from a selective decarboxylation of the mono-amino-dicarboxylic acids. Glutamic acid would give rise to γ-aminobutyric acid and aspartic would form β-alanine. Silverman (104) has observed that in ripening Cheddar, decarboxylase systems are present to a greater extent than was formerly supposed. The presence of γ-aminobutyric acid has been frequently reported.

Another matter of interest arising from the foregoing observations is the tardy appearance of proline in ripening Cheddar, and its low concentration. Silverman & Kosikowski (105) suggest that proline-containing sections of the casein molecule are more resistant to the ripening conditions pertaining to Cheddar cheese, or that proline rapidly suffers further fermentation. In this, the ripening of Cheddar differs sharply from that of Swiss Cheese, in which proline appears in much higher concentration.

During the period under review other papers have appeared dealing with the amino-acid production in the following Cheese: Bulgarian Ewe milk Cheese (106), Emmenthal (107, 108, 109, 110, 111), Czech (112), Gervais (113), Gorgonzola (114), Limburg (115), Tilsit (116).

Acidic carbonyl compounds may arise from carbohydrates and citrates, amino-acids, and from β-oxidation of fatty acids, and from these the neutral carbonyl compounds are produced. An examination of eighty-two samples of eight varieties of cheese by Bassett & Harper (117) led to the conclusion that the kind of keto-acid and its relative concentration was a characteristic of cheese variety irrespective of the age of the sample. Swiss, Cheddar and Brick cheese were characterized by keto-acids derived from carbohydrates and citrates; Blue Romano and Provolone cheese by those of fatty origin; while Camembert and Limburg contained those derived from amino-acids. Whenever a neutral carbonyl compound was present its acidic precursor was present also in appreciable quantity. In Swiss cheese (118) 80 % of the total carbonyl compounds was pyruvic acid (0·1–0·5 mg./g. cheese) and about 10 % α-keto-glutaric acid. Small amounts of α-aceto-lactic acid were present together with traces of oxal-succinic acid, oxal-acetic acid, diacetyl and acetoin. α-aceto-lactic acid appears as an intermediate product between citric acid and diacetyl. In 'blind' or eyeless Swiss cheese the pyruvic acid fell to 40 % of the total carbonyl compounds and the α-keto-glutaric acid rose to 40 %. When ^{14}C labelled sodium citrate was added to a fresh-water extract of cheese, or to a culture of *L. bulgaricus*, no radio-active pyruvic acid was formed, but α-keto-glutaric and α-aceto-lactic acids together with neutral carbonyls were radioactive. Thus, while diacetyl was produced from citric acid via α-aceto-lactic acid, the experiment showed that pyruvic acid was not formed via α-keto-glutaric acid as an intermediate. Determinations of diacetyl and acetoin in various continental cheeses are shown in Table 5 (119).

In Blue mould cheese, where the β-oxidation of fat furnishes the carbonyl compounds, Morgan & Anderson (120) were unable to establish a quantitative relationship between flavour and concentration of carbonyl compounds. Varieties examined were French Roquefort (2), Danish blue (3), Domestic blue (4) and Gorgonzola (1). The carbonyls removed by vacuum distillation were converted to their 2-4-dinitrophenyl hydrazones and separated by paper chromatography. Acetalde-

hyde, acetone, butanone-2 (methyl-ethyl ketone), pentanone-2 (methyl-propyl ketone), heptanone-2 (methyl-amyl ketone), nonanone-2 (methyl-heptyl ketone), and undecanone-2 (methyl-nonyl ketone) may be present in this type of cheese in varying amounts. Heptanone-2 and nonanone-2 were absent in one cheese with little or no aroma, but were always present together with pentanone-2 and acetone in cheese possessing a typical aroma. Only two cheeses contained traces of undecanone-2. A cheese considered to have the best aroma contained relatively little of these compounds.

Table 5

	No. of samples	mg./100 g. cheese	
		Acetoin	Diacetyl
Emmenthal	35	3·49	0·26
Ovar (Hungarian)	17	3·04	0·21
Trappist	12	2·33	0·23
Edam	13	3·67	0·14
Camembert	6	3·16	0·05
Roquefort	3	3·81	0·19

The steam distillates of various varieties of cheese were separated by Barnett & Tawab(121) using a modified gas chromatographic technique of James & Martin(122). Coulommier, Gervais, Pont l'Évêque, Dutch and Smallholder (Cheddar type) were examined. Formic acid appeared in appreciable quantity in all samples examined, the remaining volatile acids being acetic, butyric, (and on one occasion iso-butyric) and unidentified acids higher than caproic. The origin of the latter was uncertain. Lactic acid found in the early stages of ripening may suffer further transformation later in the ripening. In Smallholder cheese there was considerable fluctuation after the first week, and the authors suggest that the reappearance of lactic acid may be due to hydrolytic deamination of alanine. Total lactate in the fresh cheese ranged from 0·88 to 0·95%. Having risen to 1·59% on the seventh day in Smallholder cheese, it dropped to 1·07% on the twenty-first day and rose to 1·59% again on the twenty-eighth day. The soft cheese increased to 1·3 to 1·9% in the first week. Concentrations of formic, acetic, and butyric are shown in Table 6.

Table 6

		mg./100 g. cheese			
		Formic	Acetic	Butyric	Total
Camembert	3 days	7·7	65·7	0	73·4
Gervais	3 days	7·7	53·1	0	60·8
Pont L'Évêque	4 weeks	12·9	27·0	9·9	49·8
Dutch	2 weeks	18·2	57·6	10·0	85·8
Smallholder	2 weeks	7·8	73·8	7·5	89·1
	4 weeks	15·4	75·4	14·8	105·6
	6 weeks	15·5	85·9	22·3	123·7

Succinic, formic and/or acetic acids were present in all samples of Limburg, Gouda, Rocquefort, Gruyère and Camembert cheese examined by Simonart & Mayandon(123, 124). In Limburg cheese all the volatile acids from formic to caproic were present—particularly butyric and valeric. These two, together with caproic acid made a significant contribution to the flavour of the cheese. Lactic and acetic acids appeared after 1 week, butyric and valeric after 2 weeks, succinic, propionic and caproic after 3 weeks. Sometimes glutaric acid could be detected in this cheese. Some of the results of this work are given in Table 7.

Phenylacetic acid or benzoic acid were present in the five cheese varieties and in Limburg cheese phenylpropionic and *p*-hydroxy-phenylpropionic acids were also present. Neither Gouda nor Gruyère cheese contained *p*-hydroxybenzoic acid which was present in the other three varieties(125).

In Emmenthal cheese Lehmann & Sahli(126) found acetic and propionic acids each in a concentration of 0·25% of the whole cheese. Small, but measurable amounts of butyric acid were

also present. Although in Swiss cheese, butyric propionic and higher acids were present, there was no apparent relationship between their concentration and the age of the cheese(100). For a satisfactory flavour at least 2 mg./g. of propionic acid and 5 mg./g. proline were necessary. Provolone cheese is characterized by a relatively high concentration of butyric acid which varies according to the activity of the rennet or lipase preparations used in its manufacture(127). Acetic acid varied between 0·16–0·47 mg./g. cheese, propionic 0·09–0·66, while butyric acid varied between 0·18–4·2 mg./g. There was a fairly well-defined ratio of butyric acid to glutamic acid, namely, 1:2 which remained constant although both concentrations increased with increasing

Table 7

		m-equiv./1000 g. cheese	
		Total free acids	Total volatile acids
Limburg	3 weeks	44·8	6·5
	6 weeks	40·8	13·5
Gouda		45·6	3·5
Rocquefort		33·6	4·0
Gruyère		48·0	10·5
Camembert		9·0	1·3

flavour intensity. Some very significant differences were observed by Morris, Jezeski, Combs & Kuramoto(128) in the free-acid production of ripened blue cheese made from raw milk, homogenized milk, and pasteurized homogenized milk. The characteristic flavour appeared in 4–5 months, except in the raw milk cheese which took double the time. This cheese also showed the least concentration of caproic and higher acids: 8·52 mg./g. as compared with 20 mg./g. homogenized milk cheese and 24·93 mg./g. in the pasteurized homogenized milk cheese. The latter had the best flavour.

Methyl mercaptan, which has been detected in Limburg, Romadour, Tilsit and Dutch cheese is considered to make a contribution to flavour(129). It appears quite early in the ripening process. As mentioned in the previous review, none of the compounds identified in the steam distillates of ripe Cheddar cheese, namely: ethanol, butyraldehyde, ethyl acetate, ethyl butyrate could be held responsible for the typical flavour. Nor could any mixture of these substances alone or with diacetyl, other alcohols, aldehydes, esters or ketones simulate the typical cheese flavour(130). Fat is apparently essential for the development of the typical Cheddar flavour, for Mabbitt & Zielinska(131) found that skim-milk Cheddar merely developed a broth-like flavour. It is clear that the identification of the cheese flavour is as yet impossible. While amino-acids, volatile acids, carbonyl compounds, methyl mercaptan, and some higher acids undoubtedly make a contribution it would seem that they form a very necessary background for the actual flavouring agent. Certainly, at present, no known combination of any of these products of ripening can fully reproduce the flavour of ripened cheese.

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